Cytokines in recurrent miscarriage: Genetic and molecular studies.

LINJAWI, Sabah A.

Available from Sheffield Hallam University Research Archive (SHURA) at:
http://shura.shu.ac.uk/19965/

This document is the author deposited version. You are advised to consult the publisher's version if you wish to cite from it.

Published version


Copyright and re-use policy

See http://shura.shu.ac.uk/information.html
REFERENCE
Cytokines in recurrent miscarriage; genetic and molecular studies

Sabah Abdulaziz Linjawi

A thesis submitted in partial fulfilment of the requirements of Sheffield Hallam University for the degree of Doctor of philosophy.

December 2003
In the Name of Allah, Most Gracious, Most Merciful. Praise be to Allah, the Lord of the Worlds and blessings be upon all His Prophets and upon the last of Prophets and Messengers, Mohammad, and upon his family and his company and those who followed them with diligence up to the day of Judgement.
Acknowledgements

Firstly I would like to thank both of my supervisors, Dr Susan Laird and Dr Alex blakemore, for all their help, guidance and support throughout the whole of my PhD and special thanks are reserved for Susan, for all her time, patient assistance, continuous encouragement that I will never forget.

I would like to acknowledge the King Abdulaziz University, Jeddah, Saudi Arabia and the Saudi Attach Office, London for providing funding for this project.

Special thanks to everybody at Jessop Hospital for women for all of their help, in particular T.C. Li for provided his patients blood and biopsy samples, Lizz Tuckermann, for passing on her immunostaining skills. Thank you to everybody at Sheffield Hallam University for all of their technical support and advice. Many thanks to Naomi, Emma, Dan, and Mahmoud for their assistance in the lab and computer searching.

Special thanks also for all of my friends, both in the U.K. and in Jeddah for their sharing of the stress and putting up with me through most of this PhD.

Finally, a huge thank for my parents, my husband, my daughters and my sons for everything they have ever done for me. It is only due to their belief in me and continuous love and encouragement that I am where I am today. I dedicate this thesis to all of you.
Conferences


Oral presentation entitled "Interleukin-1 receptor antagonist and interleukin-1 beta polymorphisms in women with recurrent miscarriage".

Placenta, 9th meeting of the International Federation of Placenta Association (IFPA 2003) 24-27 September 2003 in Erbacher Hof, Mainz, Germany.

1. Presented poster entitled "GLN223ARG leptin receptor polymorphisms in women with recurrent miscarriage".

2. Presented poster entitled "Endometrial expression of IL11 and IL11R in normal fertile and recurrent miscarriage women".
CHAPTER 1
GENERAL INTRODUCTION

1.1 Female Reproductive Tract
1.1.1 The ovary
1.1.2 The uterus
1.1.3 The human endometrium
1.1.4 The menstrual cycle
1.1.4.1 The proliferative phase
1.1.4.2 The secretory phase
1.1.4.3 The menstrual phase
1.1.5 Pregnancy
1.1.5.1 The early stages
1.1.5.2 Endometrial preparation for implantation
1.1.5.3 Morphological markers of implantation
1.1.5.4 Biochemical markers of uterine receptivity
1.1.5.4.1 Adhesion molecules
1.1.5.4.2 Steroid receptors
1.1.5.4.3 Other molecules

1.2 Implantation
1.2.1 Decidualisation
1.2.2 Placentation
2.1.5 Electrophoresis
2.1.5.1 Agarose gel materials 62
2.1.5.2 Agarose gel equipment 62
2.1.5.3 Polyacrylamide gel materials 62
2.1.5.4 Equipment for polyacrylamide gel 62

2.1.6 Measuring plasma IL-1β concentration 63
2.1.6.1 Materials 63
2.1.6.2 Equipment 63

2.1.7 Preparation of PCR product DNA for sequencing 63
2.1.7.1 Materials 63
2.1.7.2 Equipment 63

2.1.8 Immunocytochemistry 63
2.1.8.1 Materials 63
2.1.8.1.1 Cryostat materials 63
2.1.8.1.2 Immunocytochemistry materials 64
2.1.8.2 Equipment 64

2.2 Methods 65
2.2.1 Human subjects 65
2.2.1.1 Peripheral blood 65
2.2.1.2 Endometrial biopsies 66
2.2.1.2.1 Normal fertile women 66
2.2.1.2.2 Women with recurrent miscarriage 66

2.3 Collection of blood 66

2.4 Collection of biopsies 66
2.5 Genomic DNA extraction
2.5.1 DNA extraction by the standard methods
2.5.2 DNA extraction by using a DNA isolation kit
2.5.3 Analysis of DNA purity and concentration

2.6 Polymerase chain reaction analysis (PCR)
2.6.1 PCR for GLN223ARG leptin receptor polymorphism
2.6.2 Genotyping GLN223ARG by restriction digest with MspI
2.6.3 PCR for IL1RN polymorphism
2.6.4 PCR for IL1B-511 C/T polymorphism
2.6.5 Genotyping IL1B-511 C/T by restriction digest with Aval

2.7 RNA extraction from cultured cells, lymphocytes and tissue
2.7.1 Extraction of RNA from cultured cells
2.7.2 Extraction of RNA from tissue samples
2.7.3 RNA extraction protocol
2.7.4 Analysis of RNA purity and concentration

2.8 mRNA expression analysis
2.8.1 Reverse transcriptase-PCR (RT-PCR)
2.8.2 First strand cDNA synthesis
2.8.3 PCR for 7B6 gene
2.8.4 PCR for interleukin-11 receptor α (IL-11Rα)
2.8.5 PCR for interleukin-11 (IL-11)
2.8.6 PCR for GAPDH
2.8.7 Nested PCR
2.8.8 Nested PCR for IL-11Rα & IL-11

2.9 Electrophoretic techniques
2.9.1 Agarose gel electrophoresis
2.9.2 Polyacrylamide gel electrophoresis
2.10 Preparation of PCR product DNA for sequencing
2.10.1 Isolation of specific DNA product
2.10.2 Automatic sequencing

2.11 Analysis of expression of IL-11Rα, IL-11 mRNA

2.12 Plasma IL-1β concentration

2.13 Linkage disequilibrium analysis

2.14 Haplotype analysis

2.15 Immunocytochemistry for IL-11 & IL-11Rα
2.15.1 Cryostat sectioning
2.15.2 Principles of immunocytochemistry
2.15.3 Immunocytochemistry protocol

2.16 Analysis of staining pattern & intensity

2.17 Statistical analysis
2.17.1 Hardy-Weinberg equilibrium
2.17.2 Pearson's chi-squared ($\chi^2$) analysis
2.17.3 Fisher's Exact Test for a 2 x 5 or smaller cross table
2.17.4 Carriage rate and allele frequency
2.17.5 F-test
2.17.6 Student's t-test
CHAPTER 3
THE GLN223ARG LEPTIN RECEPTER GENE POLYMORPHISM IN WOMEN WITH RECURRENT MISCARRIAGE

3.1 Introduction 93
3.1.1 Role of leptin in reproduction 93
3.1.2 Leptin and puberty 94
3.1.3 Leptin and menstrual cycle 94
3.1.4 Leptin and pregnancy 94
3.1.5 Leptin and recurrent miscarriage 95
3.1.6 The GLN223ARG leptin receptor polymorphism 95

3.2 Material and methods 97
3.2.1 Human subjects 97
3.2.2 Genotyping GLN223ARG leptin receptor gene 97

3.3 Results 98

3.4 Discussion 115

CHAPTER 4
INTERLEUKIN-1 RECEPTOR ANTAGONIST AND INTERLEUKIN-1BETA POLYMORPHISMS IN WOMEN WITH RECURRENT MISCARRIAGE

4.1 Introduction 118
4.1.1 The interleukin-1 system 118
4.1.2 IL-1 and endometrial function 118
4.1.3 Interleukin-1 receptor antagonist and endometrial function 119
4.1.4 Interleukin-1 receptor antagonist gene polymorphism 119
4.1.5 Interleukin-1β-511 gene polymorphism 119
### 4.2 Material and methods

- 4.2.1 Human subjects  
- 4.2.2 PCR-analysis for IL1RN and IL1B polymorphisms  
- 4.2.3 Plasma IL-1β concentration  
- 4.2.4 Electrophoresis  

**4.3 Results**

- 4.3.1 IL1RN gene polymorphism  
- 4.3.2 IL1B gene polymorphism  
- 4.3.3 Linkage disequilibrium studies of IL1RN & IL1B polymorphisms  
- 4.3.4 Haplotyping studies of IL1RN & IL1B  
- 4.3.5 Plasma IL-1β concentration measurement  

**4.4 Discussion**

- 4.4.1 IL1RN gene polymorphism  
- 4.4.2 IL1B gene polymorphism  
- 4.4.3 Plasma IL-1β levels  
- 4.4.4 Haplotype analysis  

---

**CHAPTER 5**

**EXPRESSION OF INTERLEUKIN-11 mRNA & PROTEIN IN THE ENDOMETRIUM OF NORMAL FERTILE WOMEN AND WOMEN WITH RECURRENT MISCARRIAGE**

### 5.1 Introduction

- 5.1.1 Interleukin-11  
- 5.1.2 Interleukin-11 in endometrium  

### 5.2 Materials and methods

- 5.2.1 Endometrial biopsies  
- 5.2.2 RT-PCR  
- 5.2.3 Immunocytochemistry
5.3 Results

5.3.1 Development of the PCR method to detect IL-11 mRNA expression
5.3.1.1 Optimisation of the PCR
   a) MgCl₂ concentrations
   b) Primer concentrations
   c) Numbers of the cycles
   d) Nested PCR
5.3.2 Analysis of IL-11 mRNA in human endometrium
5.3.3 Expression of IL-11 protein in human endometrium
5.3.4 Endometrial IL-11 expression in biopsies from RM women
5.3.4.1 Expression of IL-11 mRNA & GAPDH
5.3.4.2 Expression of IL-11 protein in biopsies from RM women

5.4 Discussion

5.4.1 IL-11 mRNA expression in human endometrium
5.4.2 Expression of IL-11 protein in human endometrium
5.4.3 IL-11 mRNA expression in RM women
5.4.4 Expression of IL-11 protein in RM women

CHAPTER 6

EXPRESSION OF INTERLEUKIN-11 RECEPTOR α mRNA & PROTEIN IN THE ENDOMETRIUM OF NORMAL FERTILE WOMEN AND WOMEN WITH RECURRENT MISCARRIAGE

6.1 Introduction

6.2 Material and methods
6.2.1 Endometrial biopsies
6.2.2 RT-PCR
6.2.3 Immunocytochemistry
6.3 Results
6.3.1 Analysis of IL-11Rα mRNA in human endometrium
6.3.2 Expression of IL-11Rα protein in human endometrium
6.3.3 Endometrial IL-11Rα expression in biopsies from RM women
6.3.3.1 Expression of IL-11Rα mRNA and GAPDH
6.3.3.2 Expression of IL-1Rα protein in biopsies from RM women

6.4 Discussion
6.4.1 IL-11Rα mRNA expression in human endometrium
6.4.2 Expression of IL-11Rα protein in human endometrium
6.4.3 IL-11Rα mRNA expression in RM women
6.4.4 Expression of IL-1Rα protein in RM women

CHAPTER 7
GENERAL DISCUSSION

7.1 Introduction

7.2 GLN223ARG leptin receptor gene polymorphism in RM women

7.3 IL1RN & IL1B gene polymorphisms in RM women

7.4 Interaction between IL-1 and leptin

7.5 IL-11, IL-11Rα mRNA & protein analysis in the human

7.6 IL-11, IL-11Rα mRNA & protein analysis in RM women

7.7 Overall conclusion

References
<table>
<thead>
<tr>
<th>Figure numbers</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Anatomy of the human female reproductive tract</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Maturation of human ovarian follicles</td>
<td>7</td>
</tr>
<tr>
<td>1.3</td>
<td>Anatomy of the human endometrium</td>
<td>10</td>
</tr>
<tr>
<td>1.4</td>
<td>The histology of the human endometrium &amp; myometrium</td>
<td>11</td>
</tr>
<tr>
<td>1.5</td>
<td>The menstrual cycle</td>
<td>13</td>
</tr>
<tr>
<td>1.6</td>
<td>Stages of development from fertilization to implantation</td>
<td>15</td>
</tr>
<tr>
<td>1.7</td>
<td>Implantation of the blastocyst into the endometrium</td>
<td>22</td>
</tr>
<tr>
<td>1.8</td>
<td>Radial dendrogram for the IL-1 family members</td>
<td>40</td>
</tr>
<tr>
<td>1.9</td>
<td>Structure of the VNTR of the IL1RN gene</td>
<td>46</td>
</tr>
<tr>
<td>1.10</td>
<td>Polymorphism in the gene coding for IL1B-511-C/T</td>
<td>46</td>
</tr>
<tr>
<td>1.11</td>
<td>The IL-6 family of cytokines receptors</td>
<td>48</td>
</tr>
<tr>
<td>1.12</td>
<td>Schematic diagram of leptin receptor signalling pathways</td>
<td>54</td>
</tr>
<tr>
<td>1.13</td>
<td>Schematic diagram of the position of leptin receptor polymorphisms</td>
<td>56</td>
</tr>
<tr>
<td>2.1</td>
<td>Sequence of the IL-11 gene</td>
<td>80</td>
</tr>
<tr>
<td>2.2</td>
<td>Principles of immunocytochemistry (VECTASTAIN ABC kit)</td>
<td>88</td>
</tr>
<tr>
<td>3.1</td>
<td>An agarose gel for GLN223ARG leptin receptor gene</td>
<td>101</td>
</tr>
<tr>
<td>3.2</td>
<td>Graph showing GLN223ARG leptin receptor genotypes in control and recurrent miscarriage women</td>
<td>104</td>
</tr>
<tr>
<td>3.3</td>
<td>Graphs showing GLN223ARG genotypes in recurrent miscarriage women with PCOS diseases, thyroid, coagulation or uterine abnormality</td>
<td>107</td>
</tr>
<tr>
<td>3.4</td>
<td>Graphs showing GLN223ARG genotypes in unexplained recurrent miscarriage women, unknown and retarded endometrium</td>
<td>109</td>
</tr>
<tr>
<td>3.5</td>
<td>Graph showing GLN223ARG genotype in women with primary &amp; secondary recurrent miscarriage</td>
<td>111</td>
</tr>
<tr>
<td>4.1</td>
<td>An agarose gel showing the PCR products for IL1RN gene</td>
<td>123</td>
</tr>
<tr>
<td>4.2</td>
<td>Graphs showing IL1RN genotypes in controls and RM women</td>
<td>126</td>
</tr>
<tr>
<td>4.3</td>
<td>Graphs showing IL1RN genotypes in RM women with PCOS, thyroid, coagulation or uterine abnormality</td>
<td>129</td>
</tr>
</tbody>
</table>
4.4 Graphs showing IL1RN genotypes in unexplained, unknown and retarded endometrium RM women

4.5 Graph showing IL1RN genotype in women with primary & secondary recurrent miscarriage.

4.6 A polyacrylamide gel showing the digested PCR products for IL1B gene polymorphism

4.7 Graphs showing IL1B genotype in controls & RM women

4.8 Graphs showing IL1B genotype in RM women with PCOS, thyroid, coagulation and uterine abnormality

4.9 Graphs showing IL1B genotypes in unexplained, unknown and retarded endometrium recurrent miscarriage women

4.10 Graph showing IL1B genotype in women with primary and secondary recurrent miscarriage

5.1 An agarose gel showing RT-PCR analysis of 7B6 mRNA

5.2 An agarose gel showing IL-11 RT-PCR in mRNA from epithelial cells

5.3 An agarose gel showing the IL-11 genomic DNA products

5.4 An agarose gel showing biopsies mRNA and epithelial cells mRNA

5.5 An agarose gel showing the RT-PCR products for both IL-11 & GAPDH from mRNA from normal endometrial biopsies

5.6 Graphs showing expression of IL-11 mRNA in human endometrium

5.7 Immunocytochemical staining for IL-11 in sections obtained from endometrial biopsies during the early proliferative, late proliferative, early secretory, mid-secretory and late secretory phases of the menstrual cycle

5.8 Graphs showing expression of IL-11 protein in human epithelial and stromal endometrium from normal fertile women

5.9 Immunocytochemistry staining for IL-11 in sections obtained from recurrent miscarriage biopsies during the mid-secretory phase of the menstrual cycle

5.10 Graphs showing expression of IL-11 protein in human epithelial and stromal endometrium in normal and recurrent miscarriage women
6.1 An agarose gel showing the RT-PCR analysis of mRNA from endometrial epithelial cells

6.2 The sequence results for IL-11Ra PCR product band of size 631 bp

6.3 The sequence results for IL-11Ra PCR product band of size 251 bp

6.4 An agarose gel showing the PCR product band for IL-11Ra from normal endometrium biopsies

6.5 An agarose gel showing the IL-11Ra nested PCR & GAPDH products from normal endometrial biopsies

6.6 Graphs showing expression of IL-11Ra mRNA in human endometrium

6.7 Immunocytochemistry staining for IL-11Ra in sections obtained during the early proliferative, late proliferative, early secretory, mid-secretory and late secretory phases of the menstrual cycle

6.8 Graphs showing expression of IL-11Ra protein in human epithelial and stromal cells in normal endometrial biopsies

6.9 An agarose gel showing the RT-PCR products for IL-11Ra & GAPDH from 10 recurrent miscarriage women

6.10 Graphs showing expression of IL-11Ra mRNA in endometrial biopsies from 10 recurrent miscarriage women

6.11 Immunocytochemistry staining for IL-11Ra in sections from biopsies from recurrent miscarriage women during the mid-secretory phase of the menstrual cycle

6.12 Graphs showing expression of IL-11Ra protein in human epithelial and stromal endometrium from normal and recurrent miscarriage women during the mid-secretory phase of the cycle
Tables

1.1 The frequency of the five different alleles in IL1RN gene

2.1 An example of a 2 x 2 contingency table

3.1 Concentrations of DNA and 260/280 ratios obtained from DNA from the first 102 blood samples

3.2 Concentrations of DNA and 260/280 ratios obtained from DNA from the second set of blood samples

3.3 Hardy-Weinberg analysis & Pearson's $\chi^2$ analysis of GLN223ARG polymorphism in 206 RM women

3.4 Hardy-Weinberg analysis & Pearson's $\chi^2$ analysis of GLN223ARG polymorphism in control women

3.5 GLN223ARG leptin receptor genotype in control & RM women

3.6 Distribution of GLN223ARG genotypes in RM women with PCOS, thyroid, coagulation and uterine abnormality

3.7 Distribution of GLN223ARG genotypes in RM women with unexplained RM, unknown cause and retarded endometrium

3.8 Distribution of GLN223ARG genotypes in women with primary and secondary recurrent miscarriage

3.9 The carriage rates of allele A & allele G in the RM women as a whole group or when divided according to primary or secondary RM

3.10 The carriage rates of allele A & allele G in the different sub-groups of RM

4.1 Hardy-Weinberg analysis & Pearson's $\chi^2$ analysis of IL1RN polymorphism in 206 RM women

4.2 Hardy-Weinberg analysis & Pearson's $\chi^2$ analysis of IL1RN polymorphism in controls

4.3 IL1RN genotyping in controls & the whole group of RM women

4.4 Distribution of IL1RN genotypes in RM women with PCOS, thyroid, coagulation or uterine abnormality
4.5 Distribution of IL1RN genotypes in RM women with unexplained, unknown and retarded endometrium

4.6 Distribution of IL1RN genotypes in RM women with primary & secondary RM

4.7 The carriage rate of allele 2 & allele 4 in RM women as a whole group and in primary & secondary RM women

4.8 The carriage rate of allele 2 & allele 4 in different sub-groups of recurrent miscarriage

4.9 Hardy-Weinberg analysis & Pearson’s $\chi^2$ analysis for IL1B polymorphism in 206 RM women

4.10 Hardy-Weinberg analysis & Pearson’s $\chi^2$ analysis for IL-1B polymorphism in controls

4.11 Distribution of genotypes for IL1B gene in controls & RM women

4.12 Distribution of genotypes for IL1B gene in women with PCOS, thyroid, coagulation or uterine abnormality

4.13 Distribution of genotypes for IL1B gene in RM women with unexplained, unknown or retarded endometrium

4.14 Distribution of genotypes for IL1B gene in women with primary and secondary RM

4.15 The carriage rate of allele C & allele T in RM women and in women with primary & secondary RM

4.16 The carriage rate of allele C & allele T in the different sub-groups of RM

4.17 Haplotype distribution between IL1RN & IL1B in RM and controls

4.18 Haplotype distribution between alleles of IL1RN & IL1B in primary and secondary RM

4.19 Haplotype distribution between alleles of IL1RN & IL1B in RM women with PCOS & without PCOS

4.20 The concentration of IL-1β in plasma of RM women divided according to IL1RN genotype

4.21 The concentration of IL-1β in plasma of RM women divided according to IL1B genotype

4.22 The concentration of IL-1β in plasma of RM women divided according to different sub-groups of RM
5.1 RNA concentration and ratios for RNA from endometrial epithelial cells

5.2 RNA concentration and ratios for RNA from 19 normal endometrial biopsies samples

5.3 RNA concentration and ratios for RNA from 17 RM endometrial biopsies samples

5.4 H-score values for IL-11 expression in epithelial and stromal cells from 17 human endometrial biopsies

5.5 H-score values for endometrial epithelial and stromal IL-11 expression in 16 RM biopsies & 9 normal biopsies obtained during the mid-secretory phase of the cycle

6.1 The "touch down" thermocycler programme used for IL-11Rα

6.2 H-score values for IL-11Rα expression in endometrial epithelial and stromal cells from 17 human endometrial biopsies

6.3 H-score values for IL-11Rα expression in endometrial biopsies from 16 RM & 9 normal women obtained during the mid-secretory phase of the cycle
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARG</td>
<td>Arginine</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementry DNA</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony stimulating factor-1</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CT-1</td>
<td>Cardiotropin-1</td>
</tr>
<tr>
<td>db</td>
<td>diabetes</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzadine tetrachloride</td>
</tr>
<tr>
<td>DNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>gp130</td>
<td>Glycoprotein 130</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GLN</td>
<td>Glutamine</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotrophin releasing hormone</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde phosphate dehydrogenase</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin binding epidermal growth factor</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IGF-BP</td>
<td>Insulin like growth factor binding protein</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Interleukin-1 alpha</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-1RtI</td>
<td>Interleukin-1 receptor type I</td>
</tr>
<tr>
<td>IL-1RtII</td>
<td>Interleukin-1 receptor type II</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
</tbody>
</table>
IL-4  Interleukin-4
IL-5  Interleukin-5
IL-6  Interleukin-6
IL-10 Interleukin-10
IL-11 Interleukin-11
IL-11Ra Interleukin-11 receptor alpha
IL-12 Interleukin-12
IL-18 Interleukin-18
IL-18RI Interleukin-18 receptor type I
IVF  In vitro fertilisation
icIL-1ra Intracellular form of IL-1ra
Jak  Janus kinase
Kb   Kilobase
Kda  Kilodalton
LAC  Lupus anticoagulant
LBA  Leptin binding activity
LGL  Large granular lymphocyte
LH   Leutinising hormone
LIF  Leukaemia inhibitory factor
LPD  Luteal phase defect
MMP  Matrix metalloproteinase
MAPK Mitogen activated kinase pathway
MgCl2 Magnesium chloride
mRNA messenger RNA
MUC-1 Large type 1 cell surface glycoprotein
NK   Natural killer
NaCl Sodium chloride
NaOH Sodium hydroxide
nm   nanometres
ob   obese
OB-R Leptin receptor
OSM  Oncostatin M
PBMCs Peripheral blood mononuclear cells
PBS  Phosphate buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCOS</td>
<td>Polycystic ovary syndrome</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PP14</td>
<td>Placental protein 14</td>
</tr>
<tr>
<td>Pro-IL-1</td>
<td>Intracytoplasmic 31-kDa precursors</td>
</tr>
<tr>
<td>RM</td>
<td>Recurrent miscarriage</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single strand conformation polymorphism</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SDs</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N- Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper cell type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper cell type 2</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>uNK</td>
<td>Uterine natural killer cells</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number tandem repeat</td>
</tr>
</tbody>
</table>
Abstract

Recurrent miscarriage, is defined as three or more consecutive pregnancy losses before 20 weeks of gestation. It affects 0.5-2% of pregnant women. A great variety of aetiologic factors has been identified, but a specific cause is still unknown in 50% of cases. It has been suggested that immune causes, including abnormalities in either cytokine production or immune cell populations may be the reason for some of these cases. It is also known that some women with recurrent miscarriage have an endometrial defect, which may lead to abnormal development of the feto-placental unit and subsequent miscarriage. Cytokines are known to be important in the control of embryo implantation and therefore it is possible that abnormalities in endometrial cytokine expression could provide an explanation for unexplained recurrent miscarriages. This study has therefore focused on the possible role of some pro-inflammatory cytokines and leptin in recurrent miscarriage.

In the first part of the study, polymerase chain reaction (PCR) was used to establish the frequency of alleles of IL1RN, IL1B and leptin receptor genes in the DNA extracted from peripheral blood from women who suffer recurrent miscarriage and compared to that seen in controls. The results for both IL1RN tandem repeat polymorphism and IL1B-511 polymorphism showed that there was no significant difference between the genotype distribution or allele frequency in recurrent miscarriage women and the control population. For IL1B-511 this was true whether the analysis was carried out on data obtained from the recurrent miscarriage group as a whole or when the women were divided according to the cause of the miscarriage. In the case of IL1RN polymorphism, an increased frequency of 2,2 genotype was seen in recurrent miscarriage women with PCOS, but the numbers in this group were very small.

The GLN223ARG leptin receptor polymorphism investigated in this study results in the substitution of amino acids glutamine to arginine in the transmembrane section of the receptor. The results showed no significant difference between the distribution of GLN223ARG leptin receptor genotypes in the recurrent miscarriage women as a whole group compared to the control group.
when divided according to the cause of recurrent miscarriage there was a significant increase in the AA genotype in women with secondary recurrent miscarriage and those whose aetiology is unknown.

In the second part of the study, expression of IL-11 and IL-11Rα in endometrium of recurrent miscarriage and control women were compared using RT-PCR and immunocytochemistry. The results showed that IL-11 and IL-11Rα mRNA and protein were expressed in the endometrium throughout the menstrual cycle by both stromal and epithelial cells. IL-11 and IL-11Rα protein expression was greater in epithelial cells than stromal cells. IL-11 and IL-11Rα mRNA and protein were significantly higher in the late secretory phase compared to the proliferative phase of the menstrual cycle. The high levels of IL-11 and IL-11Rα mRNA and protein in the late secretory phase suggest that IL-11 may play a role in the functional differentiation that occurs during decidualization of human endometrial stromal cells.

IL-11 and IL-11Rα were also expressed in the endometrium of women with recurrent miscarriage. There was no significant difference in amounts of IL-11Rα mRNA and protein in the endometrium obtained from normal fertile women or recurrent miscarriage women during the peri-implantation period. However, IL-11 protein expression was decreased in endometrial epithelial cells in the recurrent miscarriage women compared to that seen in normal fertile women.

Taken together these results, show decrease endometrial IL-11 production by women with recurrent miscarriage. They also suggest that leptin may be important in preventing miscarriage in some groups of recurrent miscarriage. However, further studies on large groups of recurrent miscarriage women need to be carried out in order to define the importance of this polymorphism. Although, the IL-1RN tandem repeat and IL-1B-511 polymorphisms appear not to be associated with recurrent miscarriage, this does not mean that the IL-1 system is not involved in causing recurrent miscarriage, as plasma levels of IL-1 did not appear to be different in women with different genotypes.
Chapter 1

General Introduction

Recurrent miscarriage is one of the most common complications of pregnancy, affecting 15% of pregnant women. Approximately 0.5-2% of women experience recurrent miscarriage, defined as the occurrence of three or more clinically detectable pregnancy losses (Stirrat, 1990). Despite several well established aetiologic factors, the cause of recurrent miscarriage cannot be determined in almost 50% of cases and this makes its extremely difficult to treat. It has been suggested that a proportion of these unexplained recurrent miscarriage might be due to immunologic factors (Laird et al., 2003). Abnormal endometrial development may lead to abnormal development of the feto-placental unit and subsequent miscarriage. Endometrial defect is also associated with recurrent miscarriage, and abnormal endometrial production of cytokines is known to affect embryo implantation and pregnancy outcome. Cytokines are known to play a major role in reproductive events. Certain cytokine gene polymorphisms influence the levels of cytokine production, and associations of cytokine gene polymorphisms with susceptibility to diseases and/or different clinical features/outcomes of diseases have been demonstrated (Bidwell et al., 2001). In this study we have therefore investigated the presence of IL1RN, IL1B and leptin receptor gene polymorphisms in women with recurrent miscarriage, and compared endometrial expression of IL-11 and IL-11Rα in recurrent miscarriage women and normal fertile women. Differences in expression of any of these factors might suggest a role in causing or preventing recurrent miscarriage and intervention in these pathways may therefore lead to treatments for this condition.

1.1 Female Reproductive Tract

The female reproductive tract has two distinct reproductive functions. Firstly, it must transport gametes to the site of fertilization and, secondly, provide the site of implantation for the conceptus and a suitable environment for its subsequent development. There are four components to the human female reproductive tract, the anatomy of which is shown in figure 1.1.
The ovaries are the centres of cyclicity, in that they release oocytes episodically at ovulation and are also responsible for the cyclic release of oestrogen and progesterone, two hormones that control female reproductive function. The fallopian tubes (or oviducts) consist of the fimbria, the ampulla and the isthmus, the function of which is to transport the oocytes from one of the ovaries to the uterus. The ampullary region of the fallopian tube is the site of fertilization; spermatozoa deposited in the vagina are transported up the female reproductive tract into the fallopian tube. The fallopian tube is also the site of early embryonic development as the resulting conceptus is transported to the uterus (Findlay, 1984).

The uterus is the site of implantation and placentation; it is here that growth of the embryo/fetus occurs. It consists of an upper expanded portion termed the body; a lower cylindrical part termed the cervix, and the fundus, which is the part of the body that extends above the point of entry of the fallopian tubes. The uterus is covered with a connective tissue layer called the peritoneum that extends laterally as the broad ligaments, from which the ovaries are suspended. The uterine wall consists of a thick smooth muscle layer, the myometrium and a vascular mucosal layer, the endometrium, which varies in thickness with the phases of the menstrual cycle. The final component of the female reproductive tract is the vagina, which along with the cervix is the site of deposition and capacitation of spermatozoa and is also responsible for the expulsion of the fully developed infant (Findlay, 1984).
Figure 1.1 Anatomy of the human female reproductive tract (A). Taken from Moore (1983).
1.1.1 The ovary

The ovary is covered by a layer of low columnar epithelial cells, which lines a poorly defined layer of fibrous connective tissue called the tunica albugina. Within the ovary is an inner vascular medulla containing blood vessels, which branch off to form smaller blood vessels that then penetrate into the surrounding outer cortex. The cortex of the ovary consists of stromal cells and contains the primordial follicles, which constitute the fundamental functional units of the ovary (Sadow, 1980).

At birth each ovary contains approximately $2 \times 10^6$ primary oocytes, the majority of which undergo degradation during infancy. At puberty only 40,000 oocytes remain. They are surrounded by a layer of follicular cells, which are in turn surrounded by a layer of granulosa cells. This cellular unit is surrounded by a basement membrane, the membrane propria, and is termed a primordial follicle. After puberty, a small number of follicles are recruited monthly into further growth, producing continuous development of follicles. It is not understood why there is monthly recruitment of these primordial follicles or how the follicles that do begin to develop are selected. Before an ovum can be released (at ovulation) the primordial follicle must pass through three stages of development (Findlay, 1984).

The first stage produces a primary or preantral follicle and is characterised by an increase in the size of the primary oocyte, secretion and formation of the zona pellucida by the oocyte and the development of thecal cells, produced by the condensation of some of the surrounding stromal cells. The beginning of the next phase is characterised by the formation of vacuoles in the granulosa cells, which also begin to produce a viscous fluid known as follicular fluid. As the number of vacuoles increase, a cavity called the follicular antrum is formed. This increases in the size and results in the oocyte being connected to the rim of the primordial granulosa cells by only a thin "stalk" of cells. The oocyte is however surrounded by a dense mass of granulosa cells called the cumulus oophotus (see figure 1.2).
This maturing follicle also has two distinct thecal cell layers; the inner glandular theca interna and surrounding fibrous theca externa and is now termed the secondary or antral follicle. The initiation and early progress through the preantral stage is independent of any direct extra-ovarian controls but is probably under paracrine control and cytokines may be involved (Sadow, 1980). Further on in follicular development, external support is required and this is provided endocrinologically by the pituitary gland. If there is no hormonal influence at this stage in follicular development then the follicles will undergo degradation. The presence of luteinising hormone (LH) and follicular stimulating hormone (FSH) released from the pituitary are vital in preventing degradation (Johnson and Everitt, 1995).

During the late preantral and early antral phases of follicular development receptors for these hormones appear on the follicular cells. Receptors for LH appear on the theca interna cells, whereas FSH receptors appear on the granulosa cells. The binding of LH to receptors in the theca interna cells stimulates the production of androgens from these cells and there is also a release of oestrogens (Sadow, 1980). The oestrogens are produced in one of two ways: a small amount of oestrogen is produced by de novo synthesis from acetate and cholesterol within the cell, but the majority is produced by aromatisation of androgens (Sadow, 1980). Oestrogens, progesterone and androgens are all released into the follicular fluid and oestrogens bind to receptors on granulosa cells, stimulating the proliferation of increased numbers of oestrogen receptors, therefore providing a positive feedback mechanism whereby oestrogen is stimulating an increase in oestrogen output (Sadow, 1980).

This results in the most advanced follicles providing a surge in circulating oestrogen. As oestrogen levels increase, there is an increase in LH levels caused by a positive feedback of oestrogen on the pituitary and hypothalamus. The increase in LH is rapid and results in a surge in LH that peaks approximately 18 hours before ovulation (Johnson and Everitt, 1995).
Ovulation occurs on approximately day 14 of the menstrual cycle. Because the length of the follicular phase can vary between individuals however, the exact day of ovulation is also termed day LH + 0 (i.e. no days after the LH surge). Subsequent days in the luteal phase are then referred to as day LH + the number of days after the LH surge. So if ovulation occurs on day 14 or LH+ 0, then day 20 can be referred to more exactly as day LH + 6 (Li et al., 1988).

The follicle undergoes final maturation to become a preovulatory or Graffian follicle. Oestrogen and FSH stimulate the appearance of LH receptors on the outer layer of granulosa cells. This stage is critical for the expanded antral follicle to develop into the preovulatory phase of follicular growth (Sadow, 1980). The binding of LH to receptors on the granulosa cells causes two important processes to occur. Firstly, it stimulates the terminal growth changes in the follicle and oocyte in the preovulatory follicle resulting in the oocyte rupturing through the follicle and being released, i.e. ovulation. Secondly, following the release of the oocyte, the follicle collapses. The granulosa cells are thrown into folds and bleeding occurs forming a clot in the centre. Capillaries from the theca interna infiltrate towards the centre and the granulosa cells proliferate. This whole process transforms the follicle into an endocrine organ called the corpus luteum, whose function is to produce progesterone (Johnson and Everitt, 1995).

The interval between successive ovulations is known as the ovarian cycle. The period before ovulation occurs is known as the follicular phase, due to oestrogens being secreted from the follicular cells. The period after ovulation is known as luteal phase, due to progesterone being secreted from the corpus luteum (Johnson and Everitt, 1995).
Figure 1.2 Maturation of human ovarian follicles. Taken from Findlay (1984).
1.1.2 The uterus

The human uterus is a pear-shaped organ with two uterine horns and consists of the fundus, body and cervix. The uterus is slightly flattened dorso-ventrally and thus contains a flattened uterine cavity (Sadow, 1980). The uterus consists of two layers; the myometrium (the outer smooth muscle layer) and the endometrium (the inner lining that contains numerous blood vessels and glands). The myometrium consists of bundles of smooth muscle fibres separated by vascular connective tissue.

1.1.3 The human endometrium

The endometrium is a mucous membrane lining the uterus. It is divided into three layers, the compact layer, the spongy layer and the basal layer. Its exposed surface is covered with a single layered epithelium (the luminal epithelium) that contains the tubular uterine glands. This epithelial layer is a simple columnar epithelium and is composed of a mixture of ciliated and secretory cells, and extends into the connective tissue to form glands. Beneath the surface epithelium and between the glands is a soft, cellular, connective tissue, which contains stromal cells. The stromal compartment consists mainly of stromal cells, but also includes a population of leukocytes that are dispersed throughout the stromal compartment (Bulmer et al., 1991). Also extending through the spongy layer are numerous blood vessels consisting of spiral arteries and veins. Blood supply to the endometrium is provided via the uterine artery, which is a branch of the internal iliac artery. The radial arteries cross the myometrial-endometrial junction; these are called basal arteries and are responsible for the blood supply to the stratum basalis. The stratum functionalis requires a separate blood supply as it is shed during menstruation. This is provided by the spiral arteries. Thin walled veins form an anastomosing network of sinusoids at all levels of the endometrium. Veins then follow the route of the arteries back to the uterine vein and eventually the internal iliac vein (Rodgers, 1992). Only the two layers closest to the surface participate in the glandular and other changes characteristic of the menstrual cycle and pregnancy and are thus referred to as the functional endometrial layer. Towards the end of the menstrual cycle the stromal cells undergo a morphological change and become decidual cells. These decidual cells persist through pregnancy and form the maternal part of the placenta (Tortora, 1997) (Figures 1.3 and 1.4).
The endometrium is under the control of the steroid hormones, oestrogen and progesterone. Oestrogen stimulates growth of both the myometrium and the endometrium. It also induces the synthesis of progesterone receptors in the endometrium. Progesterone is able to bring an effect after the endometrium has been primed by oestrogen. Progesterone acts on the endometrium to convert it into a hospitable and nutritious lining suitable for implantation of a fertilized ovum. Under the influence of progesterone the endometrial connective tissue becomes loose and oedematous as a result of an accumulation of electrolytes and water, which facilitates implantation of the embryo. Progesterone also induces the endometrial glands to store and secrete large quantities of glycogen and also stimulates growth of the endometrial blood vessels. Progesterone also reduces the contraction of the myometrial smooth muscle to provide a quiet environment, for implantation and embryonic growth (Sadow, 1980).

1.1.4 The menstrual cycle
The menstrual cycle consists of three phases: the menstrual phase, the proliferative phase, and the secretory or progestational phase (Figure 1.5).

1.1.4.1 The proliferative phase
This phase of the endometrium occurs while the ovary is in its follicular phase. The increasing amounts of oestradiol secreted by the developing ovarian follicles stimulate growth (proliferation) of the stratum functionalis of the endometrium. In humans spiral arteries develop in the endometrium during this phase. Oestradiol may also stimulate the production of progesterone receptors at this time. In this phase the endometrium starts to repair itself, oestrogen stimulates proliferation of epithelial cells, glands, and blood vessels, increasing endometrial thickness to 3 - 5 mm (Sherwood, 1994; Johnson & Everitt, 1995).
Figure 1.3 Anatomy of the human endometrium (B). Taken from Moore (1983).
1.1.4.2 The secretory phase

This phase occurs when the ovary is in its luteal phase. In this phase, increased progesterone secretion stimulates the development of uterine glands. As a result of the combined actions of estradiol and progesterone, the endometrium becomes thick, vascular, and spongy in appearance. The cells of the uterine glands become filled with glycogen during this phase. This endometrium is well-prepared to accept an early embryo after fertilization has occurred. This period is called progestational phase (before pregnancy) in reference to the development of the endometrium lining (Sherwood, 1994; Johnson & Everitt, 1995).

1.1.4.3 The menstrual phase

This phase occurs when fertilization and implantation do not occur. The corpus luteum degenerates, and the circulating levels of estrogen and progesterone quickly drop. Withdrawal of these steroids deprives the highly vascular-nutrient rich uterine lining of its hormonal support. The fall in ovarian hormone levels also stimulates release of uterine prostaglandins that cause vasoconstriction of the endometrium vessels disrupting the blood supply to the endometrium. The gradual reduction in O₂ delivery causes death of the endometrium, including its blood vessels. This causes bleeding through the uterine cavity together with loss of the dying endometrium tissue. The same local uterine prostaglandins also stimulate mild rhythmic contractions of the uterine myometrium. These contractions help to take the blood and endometrium debris from the uterine cavity out through the vagina as menstrual flow. Blood in the menstrual flow usually does not clot because it has already clotted and the clot has been dissolved before it passes out of the vagina (Sherwood, 1994; Johnson & Everitt, 1995).

In addition to the blood and endometrium debris, large numbers of leukocytes are found in the menstrual flow, these white blood cells play an important defence role in helping the raw endometrium resist infection. Menstruation typically lasts for about five to seven days after degeneration of the corpus luteum (Sherwood, 1994; Tortora, 1997).
Figure 1.4 The histology of the human endometrium and myometrium. Taken from Tortora (1997).
Figure 1.5 The menstrual cycle. Taken from Sherwood (1994).
1.1.5 Pregnancy
Pregnancy is a sequence of events that normally includes fertilization, implantation, embryonic and fetal growth and finally terminates in birth (Sherwood, 1994; Tortora, 1997).

1.1.5.1 The early stages
Fertilization occurs between the sperm and the oocyte at the top of the oviduct in the ampulla region of the fallopian tube to produce a single celled zygote. This cell then undergoes a series of divisions to produce more cells. During this, the total size of the conceptus remains the same. At around the 8-16 cell stage the dividing conceptus changes morphology to a compact form called a morula. At the 32-64 cell stage the blastocyst is formed. This is comprises of 2 cell types, the inner cell mass and the trophectoderm cells (Figure 1.6).

The trophectoderm cells give rise firstly to the trophoblast and then to part of an accessory fetal membrane, the chorion, which is involved in the nutrition and support of the fetus (Sherwood, 1994). The conceptus, throughout its development from the fertilization stage to the blastocyst, is enclosed within the zona pellucida. The zona has two functions; firstly it prevents the blastomeres falling apart during cleavage and secondly it prevents two genetically distinct conceptuses from sticking together to make a single chimerical conceptus composed of two sets of cells each of distinct genotype (Sherwood, 1994). The blastocyst must hatch from the zona pellucida before implantation can take place.
Figure 1.6 Stages of development from Fertilization to Implantation. Taken from Sherwood (1994).
1.1.5.2 Endometrial preparation for implantation

The human endometrium undergoes changes throughout the menstrual cycle in order to prepare for blastocyst implantation. These changes are driven by oestrogen and progesterone, which act on the endometrium and are thought to initiate a downstream cascade of molecular events via local paracrine and autocrine factors such as chemokines, cytokines, growth factors, adhesion molecules and invasive proteinases. The endometrium is only receptive to implantation for a limited period in the menstrual cycle. Psychoyos (1986) first discussed the concept of uterine receptivity and this period of receptivity is termed the "implantation window". In humans, the implantation window is approximately 4 days long, between days 20 and 24 of the menstrual cycle, or days LH+7 to LH+ 10 (Bergh and Navot, 1992).

1.1.5.3 Morphological markers of implantation

Microscopical studies have shown that endometrial epithelial cells, in particular the secretory cells, change considerably during the secretory phase of the cycle (Nikas et al., 1999a). During the early proliferative phase (days 4-7) the epithelial surface is regenerated following menstruation and is therefore very thin. Most glands are very short, straight and narrow and the stromal compartment is compact with spindle-shaped stromal cells with large nuclei. As development of the endometrium continues into the mid-proliferative phase (days 8-10) the surface epithelium becomes columnar, the glands become longer and there is evidence of stromal oedema. By the late proliferative phase (days 11-14) the glands become more tortuous showing active growth and pseudostratification of the epithelium. The stroma is moderately dense and actively growing (Noyes et al., 1950).

The secretory epithelial cells are either elongated or polygonal and vary in size. They have microvilli on their apical surface which are short and slender, which then develop with progression of the proliferative phase (Nikas et al., 1999a). In the early proliferative phase by day 16, the glands increase in diameter and tortuosity. By day 17 the microvilli are long, thick and upright, and by day 18 the microvilli begin to appear swollen. On day 19, the microvilli decrease in number and length, fuse and disappear and are replaced by smooth membrane projections that protrude and fold maximally forming pinopodes (Nikas et al.,
By day 20 there is a peak in the presence of intraluminal secretory material, microvilli are virtually absent and pinopodes are fully developed. The pinopodes only last for 24-48 hours; by day 21 microvilli reappear on the cell membranes, which are now wrinkled in appearance due to regression of the pinopodes. A possible function of pinopodes is that they absorb molecules and fluids from the uterine lumen, facilitating the proximity between embryo and endometrium. They may also be involved in trophoblast adhesion and facilitate penetration of the implanting blastocyst into the underlying stroma, because it has been noted that the lateral cell-cell contacts between epithelial cells become looser in the presence of pinopodes (Nikas et al., 1999b). From days 21-22 there is massive stromal oedema, the stromal cells increase in size and begin to appear dome-shaped. By day 23 the spiral arteries have become more prominent, indicating predecidualisation of the stromal cells. By day 24 there are collections of predecidual cells around the arterioles and stromal proliferation continues. Differentiation of the predecidua occurs around day 25 and by day 26 these cells appear as a solid sheet of well-developed decidual cells. If pregnancy has not occurred by day 24, glandular secretions become diminished and there is involution of the glandular epithelium (Nikas et al., 1999b). The glands become dilated; the previous tall, columnar epithelium become flatter. Day 27 is characterized by an increased population of polymorphonuclear leukocytes (Bulmer et al., 1991) and areas of focal necrosis and haemorrhage that become apparent a few hours preceding menstruation (Noyes et al., 1950).

1.1.5.4 Biochemical markers of uterine receptivity

1.1.5.4.1 Adhesion molecules

Biochemical markers indicating uterine receptivity include adhesion molecules and, in particular, integrins. Integrins consist of one α-subunit and one non-covalently bound β-subunit and these have been shown to be present within the endometrial epithelium (Lessey et al., 1992; Tabibzadeh, 1992). Three integrin subunits are thought to be important during the period of receptivity. These are α₁, α₄ and β₃. There is co-expression of all of these during the implantation window, but not at other times in the menstrual cycle (Lessey et al., 2000).
Expression of the $\alpha_1$ and $\alpha_4$ subunits is increased following ovulation, with a later decrease in $\alpha_1$ expression during the late secretory phase. $\beta_3$ expression is known to begin on day 19. The implanting blastocyst has the ability to modulate endometrial expression of these integrin subunits and it has been shown that the cytokine, interleukin-1 (IL-1), produced by the blastocyst mediates the up-regulation of $\beta_3$ in human endometrial epithelial cells (Simon et al., 1998).

1.1.5.4.2 Steroid receptors

Expression of progesterone and oestrogen receptors may be used as markers for endometrial maturation, as studies have found that these receptors are subject to fine hormonal control by ovarian steroid hormones (Garcia et al., 1988; Snijders et al., 1992). Receptor expression is maximal at the mid-cycle stage in response to increased oestradiol secretion during the follicular phase. Expression then declines as progesterone-dependent down-regulation occurs. During the secretory phase, the oestrogen receptor is down-regulated rapidly in the stroma and more gradually in the glandular epithelium, whereas progesterone receptors decline rapidly in the glandular epithelium, but persist in the stroma (Garcia et al., 1988; Snijders et al., 1992). These alterations in steroid receptor expression could therefore be useful as markers for uterine receptivity.

1.1.5.4.3 Other molecules

Other molecules that could be potential markers of uterine receptivity are the mucin (MUC-1) and the endometrial protein PP14 or glycodelin A. MUC-1 is a large type 1 cell surface glycoprotein with a short cytoplasmic domin (56 residues), transmembrane domin and a large extracellular domin containing a variable number tandem repeat (VNTR) sequence (Gendler et al., 1990). MUC-1 is present in both glandular and luminal epithelium during the proliferative and secretory phases of the menstrual cycle (Hey et al., 1995; Aplin, 1999). The expression of MUC-1 is up-regulated in human endometrium during the peri-implantation period (Hey et al., 1995; Meseguer et al., 2001), suggesting a possible role in implantation.
Concentrations are increased in uterine flushings from normal fertile women between days LH+7 and LH+13 (Hey et al., 1995) and there is strong expression of MUC-1 in the luminal epithelium at both the beginning and end of the uterine receptive phase (Aplin et al., 1999). Although, the exact role of MUC-1 in endometrial function is not known, it has been suggest that it may act as a uterine barrier to implantation, requiring embryonic signals before it can be removed (Aplin, 1999). As a result this may prevent any abnormal blastocysts, incapable of providing these signals, from implanting (Aplin, 1999). Recent studies suggest that MUC-1 may act as an endometrial anti-adhesive molecule that has to be locally removed by the human blastocyst in the adhesion phase (Domínguez et al., 2002; Meseguer et al., 2001). Endometrial epithelial cells also produce glycodein A (PP14), with maximal expression during the secretory phase of the menstrual cycle (Li et al., 1993). Previous studies have shown that glycodein A levels in uterine flushings correlate with the methods of endometrial dating (Li et al., 1993). Levels in endometrial flushing show similar cyclical changes to that of MUC-1 with maximal levels being present between days LH+7 and LH+10 (Dalton et al., 1995). The function of glycodein A is not known but there are some studies suggesting that it is capable of inhibiting immune reaction in vitro (Pockley et al., 1998).

1.2 Implantation

Human embryo implantation is an essential part of the reproductive process. Implantation occurs between the blastocyst and the endometrium. During the first three to four days following fertilization, the zygote remains within the ampulla, because a constriction between the ampulla and the reminder of the oviduct canal prevents any movement of the zygote toward the uterus (Sherwood, 1993).

The nutrient stored in the cytoplasm of the ovum can only sustain the zygote for less than a day. About three to four days after ovulation, progesterone is produced in sufficient quantities to relax the oviduct constriction, permitting the morula to be rapidly pushed into the uterus by oviductal contractions and ciliary movement.
The increased levels of progesterone stimulate the release of glycogen from the endometrium into the reproductive tract lumen for use as energy by the early embryo (Sherwood, 1993). When the morula arrives in the uterus, it floats freely within the uterine cavity for another three to four days. The 7 day delay after fertilization and before implantation takes place allows time for both the endometrium and the developing embryo to be prepared for implantation (Sherwood, 1993).

At the time of implantation both the blastocyst and the endometrium express various types of adhesion molecules that allow the blastocyst to adhere to endometrial cells. Implantation consists of a series of development phases in which the embryo has to appose and attach itself to the maternal endometrium and invade into it. The three phases are known as apposition, adhesion and invasion. Apposition is the orientation of the blastocyst within the lumen of the uterus. Adhesion of the blastocyst is a progressive phenomenon that ties the embryo to the luminal epithelium and is the primary event initiating an embryo-maternal relationship regulated by cytokines and adhesion molecules (Cross et al., 1994). Invasion is the process that allows the embryonic trophoblast to penetrate deep into the maternal decidua invading the endometrial spiral arteries (Blankenship et al., 1993).

The blastocyst adheres to the endometrium on its inner cell mass side. Implantation begins when the trophoblastic cells overlying the inner cell mass release proteases upon contact with the endometrium. These enzymes digest pathways between the endometrial cells, permitting finger-like cords of trophoblastic cells to penetrate into the depths of the endometrium, where they continue to digest the connective tissue between stromal cells. Also produced at this time are numerous cytokines and growth factors that are involved in the interactions between decidual tissue and trophoblast cells (Sherwood, 1993) (Figure 1.7). After the blastocyst burrows into the decidua by means of trophoblastic activity, a layer of endometrial epithelial cells covers over the surface of the hole, completely enclosing the blastocyst within the uterine lining. Attachment of the trophoblast to the endometrial epithelium is unique in that this cell-cell contact occurs via respective apical cell membranes (Sherwood, 1993).
It has been shown that these apical plasma membranes express unique cell adhesion molecules that mediate this initial attachment beginning the process of implantation.

The cytotrophoblast cells invade through the maternal decidua to the maternal arteries, where they divide into two populations, that follow different differentiation pathways. One population is the villous trophoblast cells which infiltrate the maternal blood vessels and fuse to form the syncytiotrophoblast layer. Their primary function is to form the vessels that allow exchange of oxygen and nutrients from the mother to the fetus. The second population of cells are more invasive and can invade decidua as far as the myometrium. Even in early pregnancy, these different cell types can be identified by their differential expression of various functional markers, including integrins and metalloproteinases (Bischof et al., 1997).

The initial phases of implantation occur in a non-decidualized endometrium and it is likely that the embryo dictates changes within the maternal endometrium to facilitate its own implantation (Sherwood, 1993). Implantation involves both the maternal endometrium and the embryo. It is postulated that for a dialogue needs to take place between embryo and endometrium, which may be mediated by numerous factors such as cytokines, adhesion molecules and hormones. It has been estimated that clinical implantation in the human is less than 30% efficient (Miller et al., 1980). The responsibility for this low implantation efficiency has to be shared between the embryo (30% of blastocysts are morphological abnormal at the time of implantation in vivo (Hertig et al., 1952) and defective embryonic-endometrial dialogue (30% of early pregnancy losses occurs before the expected time of menstruation). An even higher degree of early embryonic wastage occurs following assisted reproductive technologies (Simon et al., 1997).
Figure 1.7 Implantation of the blastocyst into the endometrium. (a) shows the attachment phase and (b), (c) and (d) show the stages of the invasion phase. Taken from Sherwood (1994).
1.2.1 Decidualisation

In humans, interstitial implantation occurs: the blastocyst implants deep into the maternal stroma and, as this occurs, the surface epithelium is restored above it. The blastocyst implants into undecidualised stroma; however, within a few days decidualisation occurs and extends throughout the endometrium (Bell, 1985).

Decidualisation is a differentiation of the fibroblast-like stromal cells into epithelial-like cells. In humans, a predecidual reaction will occur during the late secretory phase, even in the absence of implantation, indicating that the initial reaction is not under embryonic influence (Noyes et al., 1950). However, if implantation does occur then the reaction persists and the stromal cells become the decidua of pregnancy. During decidualisation, progesterone acts on the oestrogen primed mid-luteal phase stromal cells, causing numerous changes. The cells change shape, increase in size and there is development of organelles involved in protein synthesis. Ultrastructural studies on human decidual cells have shown that they possess all the characteristics of a secretory cell; euchromatic nucleus, numerous profiles of Golgi cisternae, dilated RER and dense, membrane-bound secretory granules (Kim et al., 1998).

Decidualisation results in localised changes in the composition of the intercellular matrix (Aplin, 1996). There is also formation of desmosomes and gap junctions between adjacent cell walls, suggesting that decidualisation could facilitate the process of trophoblast invasion (Kim et al., 1998). During decidualisation there is also an increase in endometrial leukocyte population, mainly due to an increase in large granular lymphocytes (LGLs) and, to a lesser extent, macrophages (King et al., 2000). The exact function of the decidua is unclear, but it has been postulated that the roles probably include nourishing the developing embryo, protecting the maternal tissue from excessive trophoblast invasion and protecting the embryo from immunological rejection in utero. Once the decidualisation has been completed, the decidual cells and trophoblastic cells begin to form the placenta.
1.2.2 Placentation

The placenta (Latin, *flat cake*) is disc-shaped with a diameter of about 7.5 inches and weight about 1.5 lb. when fully formed. The placenta was seen as an organ for transporting nutrients from the mother and waste from the fetus. More recently, its roles as a complex neuroendocrine organ regulating all maternal-fetal interactions, regulating the growth of the embryo and fetus, and preparing the mother for the feeding of the infant after birth have been recognized. The placenta incorporates both fetal and maternal tissue. The fetal portion develops from the chorion, which develops from the cytotrophoblast and syncytiotrophoblast (Piñón, 2002), while the maternal portion is derived from the decidua. As development proceeds, three decidual regions can be distinguished: the *decidual basalis*, marking the region in which the placenta will develop; the *decidual capsularis*, present in the early stages of implantation, but which eventually disappears; and the *decidual parietalis*, the endometrial regions far from the site of implantation. Derivatives of the chorion make contact with the decidua and form fingerlike projections known as the *chorionic villi* on its surface. The chorionic villi make the connections with the decidua and the maternal circulation (Piñón, 2002)

The fetal and maternal circulatory systems remain separate. During the embryonic period, the chorionic villi appear uniformly around the chorionic sac so that both the decidual basalis and capsularis are penetrated by them. However, beginning in the fetal period, the decidua capsularis begins to degenerate and its surface becomes smooth (*choronic laeve*). The placenta is defined by the decidua basalis (maternal portion) and the chorion frondosum (fetal portion) (Piñón, 2002). The amniotic sac, the beginnings of which go back to the formation of the blastocyst, defines the region in which the embryo and later the fetus develop. The umbilical cord connects the embryo/fetus to the placenta, and its outer covering is the amnion (Piñón, 2002).
1.3 Reurrent Miscarriage

Early pregnancy loss (or miscarriage) is defined as the termination of pregnancy before 20 weeks of gestation (dated from the last menstrual period) or below a fetal weight of 500 g and is the most common complication of pregnancy (Timbers and Feinberg, 1997). Approximately 15-20% of clinically established pregnancies abort within the first 20 weeks, usually within the first trimester (weeks 1-12) (Stirrat, 1990). The true early pregnancy loss rate is closer to 70% because of the high rate of unrecognised abortions in the first 2-4 weeks immediately following conception (Timbers and Feinberg, 1997). The majority of these are caused by chromosomal abnormalities in the sperm or the egg (Bolkage, 1990). Because a large number of miscarriages occur due to chance, investigations into the cause of miscarriages are not usually carried out until the women has been classified as suffering from recurrent miscarriage or recurrent spontaneous abortion.

“Habitual” abortion is classically defined as 3 or more consecutive abortions and affects about 1% of the female population (Timbers and Feinberg, 1997). Clinical studies have indicated that the risk of pregnancy loss after 3 consecutive abortions is 30-45% and the chance of a successful live birth after 3 consecutive abortions without a live birth is 55-60% and with at least one previous normal pregnancy (live birth) is 70% (Timbers and Feinberg, 1997). However, the diagnostic and therapeutic response to a couple with pregnancy loss is not dictated by the number of abortions, it is influenced by the woman's age, the couple’s level of anxiety, and also to factors identified in the family/medical history (Timbers and Feinberg, 1997).

There are numerous causes of miscarriage which can be grouped according to whether they relate to the embryo or the mother.

1.3.1 Parental genetic factors

One of the causes of the recurrent miscarriage is a genetic abnormality in one of the parents, and karyotyping of couples with recurrent miscarriage reveals that 3-8% have such an abnormality, most frequently a balanced chromosomal rearrangement, or a translocation (Warburton et al., 1987). Translocation and inversion are associated with pregnancy wastage (De Braekeleer et al., 1990).
Other abnormalities usually include sex chromosome mosaicism, and ring chromosomes (Timbers and Feinberg, 1997). There may also be single gene defects that are not detected by routine chromosomal analysis. Because of the difficulty in identifying these single gene defects, it is very likely that a percentage of patients considered to have unexplained recurrent miscarriage has this type of genetic defect. In addition, karyotyping of blood cells misses abnormalities of meiosis, which can only be found in sperm cell lines and ova (Timbers and Feinberg, 1997).

1.3.2 Fetal chromosomal anomalies

Several studies suggest that fetal chromosomal anomalies account for 50% of sporadic first trimester miscarriages, and possibly a similar proportion of recurrent miscarriages. Repeated sporadic fetal chromosomal anomalies occurring due to defect in meiosis, such as repetitive fetal aneuploidies due to increasing maternal age, may be responsible for recurrent miscarriage (Stirrat, 1990). One problem in the diagnosis of chromosome abnormalities is contamination of the fetal anchoring villus trophoblast with maternal cells. Therefore, a diagnosis of "46, XX" may be due to sampling of maternal tissue instead of fetal tissue (Bell et al., 1999). At present most studies use data obtained from fetal tissue that has been shown to be XY, to ensure that the tissue used for analysis is from the fetus and not the mother. Miscarriages due to abnormal karyotypes often occur before 10 weeks gestation, while miscarriages occurring after 10 weeks gestation are less likely to be associated with significant chromosomal anomalies.

It is usually thought that women over the age of 37 years are more likely to have miscarriages associated with fetal chromosomal anomalies, as results of age-related decline in oocyte quality (Li et al., 2002). However, in contrast a recent study (Ogasawara et al., 2000) found that the frequency of normal embryonic karyotypes significantly increases with the number of miscarriages, suggesting that the maternal factor of implantation and pregnancy becomes increasingly responsible for pregnancy failure as the number of previous miscarriages increases.
Two recent studies have investigated the type of chromosome abnormalities in miscarried tissue. One study of 125 miscarriage tissues from women with RM showed that only 36 (29%) had chromosomal aberrations; 94% of the aberrations were aneuploid, and 6% were structural (Carp et al., 2001). They also showed that after an aneuploid miscarriage, there was a 68% subsequent live birth compared with 41% after a euploid miscarriage. They concluded that alternative mechanisms (other than chromosomal anomalies) may be responsible for the majority of recurrent miscarriages and that patients with karyotypically aneuploid fetus have a good prognosis.

In another study (Stephenson et al., 2002), 420 miscarriage specimens from 285 couples with RM were karyotyped. Overall, 54% specimens were euploid, and the remaining 46% were cytogenetically abnormal. Among the abnormal results, 66.5% were trisomic, 19% polyploid, 9% monosomy X, 4% unbalanced translocation and 0.5% were a combination of trisomy 21 and monosomy X. In addition, the frequency of euploid miscarriages was significantly higher in women <36 years of age with RM, compared with controls, whereas in women aged ≥ 36 years there was no difference between women with RM and controls.

1.3.3 Environmental factors

Heavy coffee consumption (more than 4 cups per day) is associated with an increased risk of recurrent miscarriages (Parazzini et al., 1998). Smoking is also associated with recurrent miscarriage; the increase in risk is proportional to the number of cigarettes smoked (Dominguez-Rojas et al., 1994). There is a positive association between alcohol consumption and miscarriage, drinking >3 units per week during the first trimester increases the relative risk of recurrent miscarriage (Florey et al., 1992). Anesthetic gases and tetrachloroethylene (used in dry cleaning) have also been implicated as causative agents of miscarriage (Stirrat et al., 1990).
1.3.4 Endocrinological factors

Patients who have significant thyroid disease or uncontrolled diabetes mellitus have an increased risk of spontaneous miscarriage (Coulam et al., 1994). Few studies have examined specifically the role of diabetes mellitus in recurrent miscarriage, although there have been studies on the relationship of diabetes mellitus and sporadic spontaneous miscarriage. A review of more than 50 such studies from 1950 to 1986 found no correlation between spontaneous miscarriage and pre-conceptual or gestational diabetes (Kalter, 1987).

However, other authors have shown a significant increase in the rate of recurrent miscarriage in pregnant women with poorly controlled insulin-dependent diabetes mellitus (Miodovnik et al., 1984). In this study women with poorly controlled diabetes mellitus had a miscarriage rate of 45% in comparison with 15% in pregnant diabetic women who were controlled well. This negative effect of poorly controlled diabetes on pregnancy occurs as a result of abnormal glucose metabolism. Other studies have also shown that well controlled insulin-dependent diabetes is not associated with recurrent miscarriage (Mills et al., 1988).

Thyroid disorder as a cause of recurrent miscarriage is also controversial. The suggestion that hypothyroidism is contributory to recurrent miscarriage is derived from studies in the 1950s and 1960s. A more recent study of 219 women with recurrent miscarriage failed to detect the presence of any thyroid disease (Tho et al., 1979). However, there is some evidence that thyroid autoimmunity may be associated with recurrent miscarriage. Antithyroid antibodies have been suggested to be a predictor of pregnancy loss in randomly chosen obstetric populations since their presence had been observed to be correlated with a higher rate of recurrent miscarriage (Stagnaro-Green et al., 1990; Kilpatrick & Liston, 1995). This effect is thought to be related to abnormally activated autoimmunity rather than to over thyroid endocrine dysfunction.
1.3.5 Uterine anatomical Factors
Approximately 5-10% of women with recurrent miscarriage have a uterine malformation (Timbers and Feinberg, 1997). Uterine abnormalities can result in impaired placental vascularization during pregnancy and limited space for fetal development due to distortion of the uterine cavity. The septate uterus is the most frequent anatomical abnormality associated with recurrent early spontaneous miscarriage (Patton and Novy, 1988). Müllerian tract anomalies such as unicornuate, bicornuate, T shaped uterus, unicornuate with a rudimentary horn and submucous fibroid are also associated with recurrent pregnancy loss.

The effect of congenital Müllerian abnormalities on recurrent miscarriage is not completely understood at present, but it is possible that they may result in compromised implantation and insufficient decidual and placental growth due to poor vascularisation of the endometrium (Portuondo et al., 1986). Acquired Müllerian abnormalities include uterine synechiae (Asherman syndrome), submucosal leiomyomata and abnormalities seen following in utero exposure to diethylstilbestrol (DES).

Uterine leiomyomatas (fibroids) have varying effects on pregnancy loss depending on the size and location of the fibroid (Rock and Murphy, 1986). Different types of fibroids may affect reproductive outcome to a different extent, with submucous, intramural and subserosal fibroids being (in decreasing order of importance) a cause of pregnancy wastage (Bajekal and Li, 2000). If submucosal and myometrial myomas are large enough there are alterations in endometrial vascularisation or a possible reduction in uterine size.

Another factor that is thought to contribute to recurrent miscarriage in the late second trimester is cervical incompetence. This is characterised by painless dilation and prolapse with ballooning of the membrane into the vagina, followed by rupture of the membranes and expulsion of the immature fetus (Rock and Murphy, 1986).
1.3.6 Infections
Infections appear not to play a significant role in first trimester recurrent miscarriage. An association between recurrent miscarriage with high titres of IgG antibody to chlamydiases have been reported (Daya, 1994) but later refuted (Oser and Presson, 1996). Charles and Larsen (1990) also concluded that it is very unlikely that maternal infections cause recurrent miscarriage. A study screening for toxoplasmosis, rubella, cytomegalovirus, herpes (TORCH screen) and intrauterine swab for chlamydia carried out on 200 patients has recently been reported. None of these patients were positive for any of these agents, supporting the idea that infections do not play a major role in recurrent miscarriage (Li et al., 2002). Chorioamninitis, however, may be a cause of premature labour and mid-trimester loss.

Bacterial vaginosis is a condition associated with a complex (quantitative) alteration in vaginal flora involving Mobiluncus species, Bacteroides species, peptostreptococci and Mycoplasma hominis, and Gardnerella vaginalis. These changes are accompanied by a depletion in the number of vaginal lactobacilli. Bacterial vaginosis has been shown to be associated with an increased risk of miscarriage in the first trimester of pregnancy in women undergoing IVF treatment (Ralph et al., 1999).

1.3.7 Auto-immune disease
In autoimmunity, a humoral or cellular response is directed against a specific component of the host. The lupus anticoagulant and anticardiolipin antibodies are antiphospholipid antibodies, which arise as the result of an autoimmune disease. The lupus anticoagulant is present in a variety of clinical conditions, not just with lupus erythematosus. The antiphospholipid antibodies are directed against platelets and the vascular endothelium and cause thrombosis, spontaneous miscarriage and fetal wastage. These antibodies block prostacyclin formation, which results in unbalanced thromboxane activity leading to vasoconstriction and thrombosis. There is a strong correlation between the presence of these antibodies and recurrent miscarriage. These antibodies are also associated with fetal growth retardation and fetal death.
They are particularly associated with a high rate of second trimester fetal death (Braulke et al., 1993). The mechanism of pregnancy loss is probably decidua and placental insufficiency due to the increased tendency for blood clotting in the placental capillary network (Hadi et al., 1990).

1.3.8 Luteal phase deficit and endometrial receptivity

Other endocrine abnormalities that may cause recurrent miscarriage are luteal phase defect (LPD). This affects approximately 25% of women who suffer from recurrent miscarriage (Lee et al., 1987). Luteal phase defect refers to the abnormal development of the endometrium during the secretory phase of the cycle (Balen et al., 1993). LPD is traditionally thought of as a condition whereby progesterone production from the corpus luteum is diminished and results in inadequate endometrial development; therefore the endometrium is unable to support early pregnancy.

Histological examination of endometrial biopsy in the luteal phase is the classic method used to evaluate endometrial development. A number of studies have shown that recurrent miscarriage is associated with abnormal morphological development of the endometrium in the luteal phase, which results in the morphology of the endometrium being retarded, that is having the appearance of endometrium earlier in the cycle (Daya et al., 1988; Tulppala et al., 1991; Li et al., 2000). This may occur both in women with normal or abnormal progesterone levels i.e. may be independent of luteal phase deficit. Previous studies have also identified a correlation between endometrial function and recurrent miscarriage. Levels of the endometrial proteins PP14 (glycodelin A) and the glycoprotein MUC-1 in endometrial flushings are lower in women who suffer recurrent miscarriage (Dalton et al., 1995; Hey et al., 1995) compared to controls and in recurrent miscarriage women levels of PP14 produced by the non-pregnant endometrium correlate with pregnancy outcome (Dalton et al., 1998).
1.3.9 Unexplained recurrent miscarriage

The cause of miscarriage in 50% of women with recurrent miscarriage remains unexplained despite thorough investigations (Plouffe et al., 1992; Tulppala et al., 1993a; Clifford et al., 1994; Katz and Kuller, 1994). A number of possible aetiologies have been proposed to explain the occurrence of recurrent miscarriage in patients in whom there does not appear to be any obvious cause.

1.3.9.1 Immune mechanisms of pregnancy loss

It has been suggested that a proportion of unexplained repeated miscarriages may be due to immune mechanisms. Pregnancy in the human presents a paradox for the mother's immune system. Its primary function is to protect her body from invasion by foreign organisms and their toxic products (Sargent, 1993). To do this requires the ability to discriminate between self and non-self antigens, so that immune destruction can be targeted against the invading organism and not against her own tissues. However, these mechanisms, designed to protect the mother have the potential to destroy her antigenically foreign fetus (Sargent, 1993).

The fetus requires the trophoblast cells of its placenta to invade deep into the foreign maternal uterine tissue to establish the blood supply which is its lifeline. At the same time, it must maintain the integrity of the placenta to prevent maternal immune cells from entering the fetal compartment at a time when its own immune system is immature or absent, and is least able to defend itself from immune attack. The fact that the fetus can develop unharmed for 9 months shows that protective mechanisms must exist which permit this. These mechanisms must prevent the immune rejection of the fetus while maintaining the mother's ability to fight infection (Sargent, 1993).

1.3.9.2 Immune cell-populations

The population of leukocytes in human decidua and endometrium is distinctly different to that of peripheral blood. In endometrium there are no B cells (Bulmer et al., 1991; Johnson et al., 1999) and very few neutrophils (Salamonsen and Lathbury, 2000).
The endometrium population consists mainly from three cell types; T cells, macrophages and uterine natural killer cells (uNK), which are also called large granular lymphocytes (Bulmer, 1995; 1996; Johnson et al., 1999). The endometrial NK cells are different from the NK cells found in the peripheral blood. The uterine NK cells express CD56 and CD38, but not the classical T-cell or NK-cell markers CD3, CD4, CD8, CD16 and CD57 (Bulmer et al., 1991). A small proportion of uNK cells are similar to the peripheral NK cells and show minimum expression of CD56, and are referred to as CD56\textsuperscript{dim} cells, while the major population of uNK cells are called CD56\textsuperscript{bright} cells.

T-cells make up about 45% of endometrial leukocytes in the proliferative phase of the cycle. Their absolute numbers do not change throughout the menstrual cycle and in early pregnancy, but their relative numbers decrease (Bulmer et al., 1991). The macrophages make up 15-20% of endometrial leukocytes, and their numbers increase slightly during the secretory phase of the cycle and early pregnancy (Bulmer, 1995).

The most dramatic change in leukocyte numbers is seen for the CD56+ uNK cells. In the proliferative phase of the cycle, CD56+ uNK numbers are equal to T cells numbers, but their numbers increase during the secretory phase of the cycle and during the mid-secretory phase of the cycle they make up 70% of the endometrial leukocytes. Their numbers increase further during early pregnancy (King et al., 1989; Bulmer et al., 1991). This increase in number suggests that they are important in menstruation and early pregnancy and it is thought that they may play a role in preventing the maternal rejection of the fetus.

1.4 Cytokines

Cytokines are polypeptides involved in the control of immune processes. They may therefore be involved in controlling the maternal immune enviroment and, thus, in preventing pregnancy loss. Cytokines have been divided into families depending upon the immune cell of origin and the immunological effects that they produce. The major immune cells involved in cytokine production are CD4+ T-helper cells, which can be divided into three functional subsets based on their cytokine production. Firstly, Th1 cells produce interferon gamma (IFNg), IL-2 and tumour necrosis factor beta (TNF\beta). These are the main effectors of
cell-mediated immune responses. Secondly, Th2 cells, which produce IL-4, IL-5, IL-6 and IL-10, and these are the main effectors of antibody-mediated humoral responses. The third T-helper cell population is that of the Th0 cells; these are precursor cells which can be converted to either Th1 or Th2 type cells and can produce both Th1 and Th2 cytokines as well as TNFα and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Lim et al., 2000). A further family of cytokines are the pro-inflammatory cytokines, such as IL-1, TNFα, IL-6 and leukaemia inhibitory factor (LIF). These are produced by macrophages and are involved in the inflammatory events associated with tissue damage and repair. It is known that all these cytokines are also produced by cells other than immune cells, including the epithelial and stromal cells of the endometrium and the decidua and cytotrophoblast cells of the placenta (Laird et al., 2003).

### 1.4.1 Cytokine receptors

Cytokines are proteins/glycoproteins and cannot pass through the plasma cell membrane. They must, therefore, exert their cellular functions via interactions with specific membrane-bound receptors. Generally, cytokine receptors consist of three domains. Firstly, the extracellular domain, which provides the binding site for the cytokine and also creates specificity for that particular ligand. Next is the transmembrane domain, which spans the phospholipid bilayer of the plasma membrane, and finally the intracellular or cytoplasmic domain that has either enzymatic activity or binds other molecules in order to deliver a signal inside the cell in response to binding of the cytokine ligand. The extracellular domains of cytokine receptors can be cleaved to produce soluble forms of the receptor (Hilton, 1994). Most cytokine receptors signal through another molecule and this signalling molecule is often used by more than one cytokine. For example, gp130 is the signalling molecule used by all members of the IL-6 family of cytokines, including LIF, IL-11, oncostatin M and IL-6 itself (Taga, 1997).

### 1.4.2 Cytokines and pregnancy

The presence of numerous cytokines at the maternal-fetal interface has been shown and the receptors for these cytokines are present on trophoblast cells, suggesting that they may play a role in the growth and survival of the fetoplacental unit (Sharkey, 1998).
The exact role of cytokines in maternal-fetal interaction is still poorly understood, particularly in humans. Experiments in mice have suggested the importance of Th2 cytokines in successful pregnancy outcome (Wegmann et al., 1993). Th1-type cytokines have been shown to be detrimental to pregnancy outcome. TNFα and IFNy inhibit the implantation of the mouse embryo. They have also been shown to inhibit the proliferation of and stimulate apoptosis of human trophoblast cells in vitro (Hill et al., 1991). IL-10, a Th2-type anti-inflammatory cytokine, prevents fetal wastage in mice prone to fetal resorptions and, conversely, the administration of anti-IL-10 antibodies increases fetal wastage (Chaouat et al., 1995). These results suggest that Th1 based reactivity is associated with pregnancy failure and normal pregnancy is biased in favour of a Th2-dominant status (Wegmann et al., 1993; Raghupathy et al., 1997).

Further work in animals has suggested the importance of other cytokines in regulating embryonic implantation and development of the feto-placental unit (Cross et al., 1994). Mutations in the maternal colony stimulating factor-1 (CSF-1) gene in the osteoporotic mutant mouse compromise implantation (Polland et al., 1991). It has also been shown that blastocyst implantation depends on the uterine expression of leukaemia inhibitory factor (LIF) in mice (Stewart et al., 1992). Implantation does not occur in LIF knock-out mice, although transfer of homozygous LIF-negative blastocysts to pseudopregnant, wild-type mice results in normal implantation and pregnancy outcome (Stewart et al., 1992). Blockade of endometrial interleukin-1 receptor type 1 (IL-1R1) by its natural antagonist interleukin-1 receptor antagonist (IL-1ra) also prevents implantation in mice (Simon et al., 1994). Studies in mice showing that IL-11Ra is essential for normal decidual development have also suggested that IL-11 is important in implantation and pregnancy (Bilinski et al., 1998; Robb et al., 1998). Female mice with either an inactive or null mutation for the IL-11 receptor α chain (IL-11Ra) are fertile, and their blastocysts implant and elicit an initial decidual response. However, only small decidua form and then subsequently degrade to result in pregnancy loss (Bilinski et al., 1998; Robb et al., 1998).
1.4.3 Th1 & Th2 cytokines in recurrent miscarriage in humans
Although the evidence for the importance of Th1/Th2 cytokines balance in successful pregnancy outcome in mice is strong, there is less evidence for it in humans. Previous studies reported that the predominant maternal immune response during pregnancy is humoral rather than cell-mediated (Wegmann et al., 1993). Cell-mediated autoimmune diseases such as rheumatoid arthritis are improved during human pregnancy, while antibody-mediated diseases such as systemic lupus erythematosus are exacerbated, which indicates an association with a down-regulation of Th1-type activity and an enhancement of Th2-type reactivity.

Several studies have shown alterations in Th1 and Th2 cytokine production in women with recurrent miscarriage. It has been shown that peripheral blood mononuclear cells (PBMCs) taken before pregnancy from women with recurrent miscarriage produce TNFα and IFNγ in response to stimulation from trophoblast cell extracts, while cells from non-pregnant women with normal reproductive histories and from men produced IL-10 in response to incubation with trophoblast cell extracts (Hill et al., 1995). Further studies have shown that this abnormal Th1-type response is not seen in women with recurrent miscarriage with chromosomally abnormal fetuses, or in women with a uterine structural abnormality (Hill et al., 1995). The supernatants taken from PBMCs prepared from recurrent miscarriage women were toxic to mouse embryos and this has been shown to be due to the presence of IFNγ in the supernatants.

Recent studies have shown decreased production of IL-4, IL-5, IL-6 and IL-10 and increased production of IFNγ, IL-2, TNFα and TNFβ in stimulated PBMCs obtained from women with recurrent miscarriage compared with stimulated PBMCs obtained from normal women (Raghupathy et al., 1999; 2000). However, in these studies results were compared either at the time of miscarriage and no miscarriage or during the first trimester and at birth, and these different studies may affect the results. Hamai et al. (1998) has also show that unstimulated PBMCs isolated from women with unexplained recurrent miscarriage produce more IL-2 than either unstimulated PBMCs from normal women, or males (Hamai et al., 1998). However, a recent study has shown increased IL-4 and IL-10 and decreased IFNγ production by peripheral blood
cells in women with recurrent miscarriage (Bates et al., 2002) compared to controls. In contrast to other studies, the measurements in the recurrent miscarriage women in this study were taken in early pregnancy, prior to the miscarriage event. Another study has reported significantly lower levels of IL-4 and IL-10 produced by CD4+ T cell clones isolated from decidua obtained from recurrent miscarriage women compared to that produced by CD4+ cells prepared from decidua from normal women (Piccinni et al., 1998).

Th1 and Th2 cytokine mRNA expression in the endometrium of normal fertile women and recurrent miscarriage women during the peri-implantation phase of the menstrual cycle has also been investigated (Lim et al., 2000). This study showed that fewer women with recurrent miscarriage had detectable levels of IL-6 in their endometrium, but more had detectable levels of TNFα, IFNγ, IL-2 and IL-12, compared with control fertile women.

1.4.4 Endometrial expression of pro-inflammatory cytokines
Recent studies from our laboratories have used immunocytochemistry in women with recurrent miscarriage to investigate the expression of IL-1α, IL-1β, LIF and IL-6 protein in the endometrium of women with recurrent miscarriage and compared that with the expression in normal fertile women (Cork et al., 1999). LIF, IL-6, IL-1α and IL-1β immunostaining was decreased in endometrium from recurrent miscarriage women compared with staining in endometrium from normal fertile women. Other studies have also reported decreased IL-1β and IL-6 mRNA expression in the endometrium of women with RM (von Wolff et al., 2000).

1.5 The interleukin-1 system
IL-1 is an inflammatory cytokine that has been implicated in mediating both acute and chronic pathological inflammatory diseases. Two functionally similar molecules, IL-1α and IL-1β, are encoded by separate genes (respectively, *IL1A* and *IL1B*). The third gene of the family (*IL1RN*) encodes IL-1 receptor antagonist (IL-1ra), a non signalling anti-inflammatory molecule that competes for receptor binding with IL-1α and IL-1β (Nicklin et al., 2002). All three proteins bind the only functional signalling receptor for IL-1, the type 1 IL-1 receptor (*IL1R1*) (Sims et al., 1993).
The classical IL-1 family genes *IL1A*, *IL1B*, and *IL1RN* were mapped by recombination analysis to the same chromosomal region in humans (Steinkasserer et al., 1992). The genes were shown to be clustered, and the maximum separation of the distal genes *IL1A* and *IL1RN* was estimated to be 430 kb (Nicklin et al., 1994). The cytogenetic map position of the cluster was found to be 2q13 (Nothwang et al., 1997). IL-18 is the fourth member of the IL-1 structural family (Bazan et al., 1996). It is also a proinflammatory cytokine, and its signal transduction mechanism shares many components with that of IL-1. IL-18 does not bind to IL1R1, but to a related receptor (IL-18R1) (Torigoe et al., 1997). Over the past two years, various groups reported putative proteins that contain IL-1-like elements (Mulero et al., 1999; Smith et al., 2000; Kumar et al., 2000). Six new genes have recently been agreed by all of the investigators involved in the discovery of the genes (Sims et al., 2001). The new human genes have been named *IL1F5*, *IL1F6*, *IL1F7*, *IL1F8*, *IL1F9*, and *IL1F10* (Figure 1.8).

Molecular cloning of *IL1A* and *IL1B* genes has revealed a nucleic acid homology of 45% (Veerapandian et al., 1992). *IL1A* and *IL1B* genes are differentially expressed in various tissues (Kobayashi et al., 1989). The primary amino acid sequence of mature IL-1β is conserved among the various animal species in the range of 75-78% whereas the α-sequence of IL-1α is conserved in the range of 60-70% (Dinarello et al., 1988).

The two forms of IL-1 are initially synthesized as intracytoplasmic 31-kDa precursors (pro-IL-1). ProIL-1β is cleaved by IL-1β converting enzyme, (Cerreti et al., 1992) generating a carboxyl-terminal 17-kDa peptide called "mature" IL-1β which is the biologically active form, and is released by the cell to the extracellular space and the circulation. IL-1α precursor is transported to the cell surface and anchored to the plasma membrane. The precursor forms of IL-1α and IL-1β are not secreted, but instead accumulate in the cytoplasm. The active form of IL-1α is not secreted, whereas active IL-1β is secreted (Mosley et al., 1987).
IL-1 receptor antagonist (IL-1ra) is a 22-25-kDa protein that is related structurally to IL-1α and IL-1β, and is one of the most powerful endogenous anti-inflammatory agents known. It competes with IL-1α and IL-1β for occupancy of cell surface receptors, but does not stimulate signal transduction, thus acting as an inhibitor of IL-1 action (Arend et al., 1991; Dinarello et al., 1991). Two forms have been described: the secreted form as produced by monocytes, and the intracellular (keratinocyte) form, which although encoded by the same gene, is transcribed from an upstream promoter with an alternative first exon (Haskill et al., 1991).
Figure 1.8 Radial dendrogram showing the most probable tree for the evolution of all known IL-1 family members, branch lengths indicate evolutionary distance. Taken from Nicklin et al. (2002).
1.5.1 Interleukin -1 receptors

There are two IL-1 receptors, now called IL-1 receptor type I and II (IL-1Rtl and IL-1RtII) with molecular weights of 80 kDa (Sims et al. 1988) and 64 kDa (Horuk et al. 1989) respectively. Both are members of the immunoglobulin super-family and are structurally related to each other. Within the immune system, the type I receptor is found primarily on T cells, endothelial cells, and macrophages, whereas the type II receptor is found on neutrophils, B cells, and bone marrow cells. Some cells, such as monocytes, probably express both types and both may cooperate in binding and signal transduction. When the two IL-1R types are present in the same cell, they function independently at the level of ligand binding, and do not form a heterodimeric receptor complex (Slack et al. 1993).

IL-1Rtl consists of 569 amino acids made up of a 20 amino acid signal peptide, a 317 amino acid extracellular domain, containing three immunoglobulin-like domains, a 22 amino acid transmembrane domain and a 210 amino acid intracellular domain. The gene encoding IL-1Rtl is situated on chromosome 2 at position 2q12 and transcribes a 5Kbp mRNA. IL-1RtII consists of 398 amino acids, consisting of a 13 amino acid signal peptide and a 334 amino acid extracellular domain, which shares 28% homology to the amino acid sequence of the IL-1Rtl extracellular domain. It consists of a 22 amino acid transmembrane domain and a 29 amino acid intracellular tail, both of which show no similarity to these regions of the IL-1Rtl. The lower molecular weight is due to a shorter cytoplasmic region. The gene encoding IL-1RtII is situated on chromosome 2 at position 2q12-2q13 (Sims and Dower, 1994).

Both IL-1α and IL-1β act as agonists for both receptor types, whereas IL-1ra acts as an antagonist for both receptors, possibly providing a mechanism whereby the biological effects of IL-1 can be suppressed. The available information indicates that IL-1RtII acts as a decoy receptor, and so acts as another inhibitor of IL-1 activity. IL-1α and IL-1β can bind to IL-1RtII but no signal is produced because the intracellular domain of this receptor is much shorter than that of IL-1Rtl.
Signalling through IL-1RI involves the interaction between the cytoplasmic Toll-like domain of the receptor (Heguy et al., 1992). IL-1ra differs from IL-1α and IL-1β in failing to activate the interaction between IL-1R1 and the second receptor component, IL-1 receptor accessory protein (IL-1RACP). This is a transmembrane protein that is a distant relative of IL-1R1, having a similar domain structure, but it has no intrinsic affinity for IL-1 (Greenfeder et al., 1995; Wesche et al., 1997)

All the mature forms of IL-1 have affinity for both receptors, although IL-1ra binding has the highest affinity for IL-1Rt1 and IL-1β binding has highest affinity for IL-1Rtill. The precursor form of IL-1β does not bind to IL-1Rt1, which explains why proIL-1β has no biological activity. However, the receptor binding and biological activities of the mature and pro forms of IL-1α appear indistinguishable (Mosley et al., 1987). Although, the effects of both IL-1α and IL-1β on IL1Rt1 are similar, IL1β may be of greater biological significance because only it is secreted from the cell.

1.5.2 IL-1 in the endometrium and decidua

The IL-1 family of cytokines has been shown to be expressed by the human endometrium and they may have a role to play in implantation. Fukuda et al. (1995) demonstrated, using ELISA and RT-PCR, that all three major members of the IL-1 family of cytokines were expressed by human endometrial cells in vitro. The presence of both forms of IL-1 agonist (IL-1α and IL-1β) has been demonstrated in human endometrium, at the mRNA and protein levels. mRNA expression is seen in endometrial macrophages and endothelial cells, as well as in epithelial and stromal cells (Tabibzadeh and Sun, 1992). Other studies have also shown IL-1α, IL-1β and IL-1ra protein in epithelial and stromal cells in vivo (Simon et al., 1993a; Simon et al., 1994a; Simon et al., 1995a).

IL-1Rt1 has been shown to be present in the human endometrium throughout the menstrual cycle (Simon et al., 1993a; Simon et al., 1993b) and also within decidual glands and syncytiotrophoblast cells (Simon et al., 1994a). Maternal decidua and villous cytotrophoblast and syncytiotrophoblast cells are also known to express IL-1β and IL-1ra (Simon et al., 1994a).
*In vitro* studies have also shown that IL-1 is capable of affecting endometrial and decidual cell function. It stimulates epithelial and stromal IL-6 production (Tabibzadeh et al., 1989; Laird et al., 1994; Vandermolen and Yang, 1996) and endometrial and decidual LIF production (Arici et al., 1995; Sawai et al., 1997). IL-1 has also been shown to stimulate MMP production by both endometrial and cytotrophoblast cells (Simon et al., 1998; Sharkey et al., 1998). Successful implantation after *in vitro* fertilization has been correlated to high concentrations of both IL-1α and IL-1β in the culture media of human embryos (Sheth et al., 1991; Baranao et al., 1997; Karagouni et al., 1998).

### 1.6 Gene polymorphism's

Gene polymorphism refers to the stable simultaneous occurrence in the population of genomes showing allelic variations at a level too high to be accounted for by somatic mutation alone. They are central to medical genetics and the identification of the disease-causing genes in many disorders has been possible through polymorphism analysis. Variation in DNA sequence can result from a number of different genetic changes including single-base substitutions, variable copy number of repeat sequences, insertions and deletions (Tarlow et al., 1993).

Single base changes can be detected in a number of ways. Firstly, if a restriction enzyme site is affected then the pattern of bands after digestion with that enzyme will be different and can be detected by southern blots or by polymerase chain reaction (PCR) followed by digestion of the product with a restriction endonuclease to produce allele-specific fragments. These are known as restriction fragment length polymorphism's (RFLP).

Secondly, an altered base may affect the migration of short fragments of single-strand DNA due to alterations in the secondary structure. These can be seen by PCR amplification of the region of interest followed by denaturation of the two strands, which can then be separated on a polyacrylamide gel. This technique is known as single strand conformation polymorphism (SSCP).
1.7 Interleukin-1 receptor antagonist gene polymorphisms (IL1RN)

The IL1RN gene is located on the long arm of human chromosome 2, close to the related genes encoding IL-1α and IL-1β (Nicklin et al., 1994). Previous studies have reported a variable-length polymorphism in intron 2 of the IL1RN gene (Figure 1.9) (Tarlow et al., 1993). The IL1RN polymorphism results from a variable copy number of an 86-base pair tandem repeat (a VNTR polymorphism). 5 alleles have been found in this system, representing 2, 3, 4, 5, and 6 copies of the repeat sequence (Tarlow et al., 1993). The 4-repeat (IL1RN*1) and 2-repeat (IL1RN*2) alleles are relatively common in European populations, while 3-repeat and 5-repeat alleles occur at much lower frequencies, and the 6-repeat allele is very rare (Table 1.1).

Several studies have found an association between the IL1RN*2 allele and various diseases, including psoriasis (Cork et al., 1993), ulcerative colitis (Mansfield et al., 1994), diabetes mellitus (Blakemore et al., 1994), systemic lupus erythematosus (Blakemore et al., 1994), rheumatoid arthritis (Buchs et al., 2001), alopecia areata and coronary heart disease (Tazi-Ahnini et al., 2002). An association between IL1RN*2 and recurrent miscarriage has been reported recently among 105 Austrian women compared to 91 controls (Unfried et al., 2001).

1.8 Interleukin-1 beta gene polymorphisms (IL1B)

The gene encoding IL1B is located within a 40 kb region on chromosome 2q14.2 (Bioque et al., 1995). Three different polymorphisms have been described; two in the promoter region, at position -511 and -31 and, one in exon 5 at position +3954 (Figure 1.9). The polymorphisms at positions -511 and -31 are substitutions of cytosine by thymine, while the polymorphism at exon 5 is created by G to A base substitution at position +3954. The exon 5 polymorphism of the IL1B gene has been shown to be in linkage disequilibrium with the -511 polymorphism (di Giovine et al., 1997).
Table 1.1 The frequency of the five different alleles present in IL1RN gene (Tarlow, et al., 1993).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
<th>Approximate size (bp)</th>
<th>Number of repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1</td>
<td>0.736</td>
<td>410</td>
<td>4</td>
</tr>
<tr>
<td>A 2</td>
<td>0.214</td>
<td>240</td>
<td>2</td>
</tr>
<tr>
<td>A 3</td>
<td>0.036</td>
<td>500</td>
<td>5</td>
</tr>
<tr>
<td>A 4</td>
<td>0.007</td>
<td>325</td>
<td>3</td>
</tr>
<tr>
<td>A 5</td>
<td>0.007</td>
<td>595</td>
<td>6</td>
</tr>
</tbody>
</table>
Some studies have shown that the presence of different alleles of IL1B polymorphisms are associated with the differences in plasma levels of IL-1β (Helfer et al., 2001). The presence of the IL1B-31T and IL1RN*2/*2 alleles have been associated with increased risk of hypochlorhydria induced by both H. pylori infection and gastric cancer (El-Omar et al., 2000). The IL1B (+3954) rare allele (allele 2) has been related to an increased risk of rheumatoid arthritis (Cantagrel et al., 1999). Other studies have confirmed the correlation between the IL-1B-511 and IL-1B+3954 polymorphisms and disease severity in rheumatoid arthritis (Buchs et al., 2001), ulcerative colitis (Pociot et al., 1992), insulin-dependent diabetes (Stokkers et al., 1997), myasthenia periodontitis (Huang et al., 1998).

There are also some studies which have investigated these IL1B polymorphisms in women with recurrent miscarriage. Two studies have investigated the exon 5 polymorphism and shown no association between either allele of the exon 5 polymorphism and recurrent miscarriage (Reid et al., 2001; Helfer et al., 2001). In the Helfer study, serum IL-1β levels were also measured and were shown not to be related to the genotype. Other studies have shown no significant association between recurrent miscarriage and alleles at the -308 position of the TNFA gene (which codes for TNF-α), the +874 position of the IFN-gamma (which encodes IFN-γ) or the -1082 position in the IL-10 gene promoter region in women with recurrent miscarriage and normal fertile women (Babbage et al., 2001; Reid et al., 2001).
Figure 1.9 Structure of the VNTR (86 bp tandem repeat) of the IL1RN gene. Arrows indicate the positions of the primers used for PCR amplification of intron 2 from genomic DNA. The white boxes represent non-translated regions of the exons, the black boxes represent translated regions.

Figure 1.10 Polymorphism in the gene coding for IL-1β-511-C/T on chromosome 2q14.2.
1.9 Interleukin-11 (IL-11)

IL-11 belongs to the IL-6 family of cytokines that includes IL-6, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotropin-1 (CT-1) (Figure 1.11). It is known to have a number of biological activities on a number of different cell types. The mature protein is a monomer of 179 amino acids and has a molecular weight of 19 kDa. It is an important stimulator of haematopoiesis (Musashi et al., 1991). It also induces B cell differentiation (Yonemura et al., 1992), stimulates osteoclast development, induces synthesis of acute phase protein in hepatocytes and inhibits adipocyte differentiation (Kawashima et al., 1991), modulates extracellular matrix metabolism and induces IL-6 mRNA synthesis in T-helper cells and monocytes (Du and Williams, 1997).

Interleukin-11 was first identified as a soluble factor released by PU-34 primate bone marrow stromal cells. It has the ability to stimulate the proliferation of interleukin-6 (IL-6)-dependent cells (Paul et al., 1990). Human IL-11 cDNA was later cloned from a fetal lung fibroblast cell line (Paul and Schendel, 1992). The IL-11 precursor is 199 amino acids in length and has a molecular mass of 23kDa (Trepicchio and Dorner, 1998). IL-11 has a similar four-alpha-helix bundle structure to IL-6 and LIF. It lacks any glycosylation sites or cysteine residues, but is a highly stable molecule (Du and Williams, 1997). The gene for human IL-11 is 7Kbp in size, contains 5 exons and 4 introns and is located on chromosome 19q13.3-19q13.4. Transcription gives rise to two mRNA transcripts of 2.5Kbp and 1.5Kbp (Paul et al., 1990; Du and Williams, 1997), the difference resulting from the presence of additional 3’ noncoding sequences within the larger transcript.
Figure 1.11 The IL-6 family of cytokines receptors. Members of the IL-6 family of cytokines and the components of their receptor complexes. Taken from Robb et al. (2002).
1.9.1 Interleukin-11 receptor alpha (IL-11Rα)

IL-11 brings about its effect in cells by binding to a plasma membrane receptor which is comprised of two subunits, IL-11Rα and gp130. The IL-11Rα subunit binds IL-11 specifically, while gp 130 has no affinity for IL-11. IL-11Rα is a 422 amino acid protein with a molecular weight of 150 kDa. Its gene spans a region of 9Kbp, consisting of 12 exons and 12 introns and is located on chromosome 9p13 (Du and Williams, 1997). The human interleukin-11 receptor-α chain (IL-11Rα) is a membrane-anchored glycoprotein that is also widely expressed in various tissues and cells (Chérel et al., 1995; Robb et al., 1996; Nandurkar et al., 1997).

The gene encoding the human IL-11Rα chain has been cloned from Caucasian female placenta, shown that it is present in two isoforms; IL-11Rα 1 and IL-11Rα 2 (Chérel et al., 1995). IL-11Rα 1 has a small intracytoplasmic domain comparable to that of the IL-6 receptor α chain (IL-6Rα) (Yamasaki et al., 1988), while IL-11Rα 2 lacks this domain and may be linked to the membrane by a glycosphatidylinositol linkage as described for the ciliary neurotrophic factor receptor α chain (CNTFRα) (Davis et al., 1991).

As with other members of the IL-6 family of cytokines, the signal of binding of IL-11 to its receptor is transmitted to the cytoplasm of the cell via the gp130 signalling molecule. Binding of IL-11 to its receptor results in recruitment of gp130. This is followed by dimerization of the gp130-IL11Rα units. gp 130 has been shown to activate a number of different signalling pathways. Jak 1, Jak 2 (Janus activator kinases) and Tyk 2 have been implicated in signalling from gp130, together with STAT 1 and STAT 3 (signal transducers and activators of transcription) (Karpovich et al., 2003). Recruitment of Jaks to the activated gp130 molecules results in phosphorylation of tyrosine residues in the gp130 cytoplasmic domain which then allows STATs to bind. The phosphorylated STAT factors from homo- or heterodimers, translocate to the nucleus and bind to the promoter region of responsive genes (Yin and Yang, 1994; Yin et al., 1994; Yang and Yin, 1995). gp 130 signalling is also postulated to occur via the ras / raf / MAPkinase pathways (Hibi et al., 1996).
1.9.2 IL-11 and endometrial function

There are several studies showing the expression of IL-11 and IL-11R in both the mouse and human endometrium. The expression of IL-11 in the mouse endometrium is maximal during decidualization, suggesting that IL-11R interactions in the decidua are important in this process (Robb et al., 1998). However, recent studies have reported that IL-11 impairs decidualization in pregnant rats, and causes abnormal development of both the embryo and trophoblast (Caluwaerts et al., 2000). More recent studies have shown that low doses of IL-11 improve hatching and attachment of blastocysts, but both high and low doses impair decidualization in rats (Caluwaerts et al., 2002).

IL-11, IL-11Rα and gp130 are also expressed in the human endometrium (Dimitriadis et al., 2000; Cork et al., 2001; Cork et al., 2002). IL-11 is expressed by both stromal and epithelial cells, but expression by epithelial cells is greater than by stromal cells (Cork et al., 2001; Dimitriadis et al., 2000). Endometrial epithelial expression of IL-11 is greater in the late proliferative and early secretory phases of the cycle than at other times (Cork et al., 2001). Stromal expression of IL-11 also increases dramatically at the end of the cycle and during decidualisation (Dimitriadis et al., 2000; Cork et al., 2001). IL-11 production by both stromal and epithelial cells in vitro is increased by IL-1, TNFα and TGFβ (Cork et al., 2001). Stromal cells production of IL-11 is also stimulated by heparin binding epidermal growth factor (Karpovich et al., 2003). In turn, IL-11 is reported to decrease TNFα production by endometrial epithelial cells in vitro (Cork et al., 2002). IL-11 has also been shown to be involved in the functional differentiation that occurs during progesterone-mediated decidualisation of human endometrial stromal cells in vitro (Dimitriadis et al., 2002).

Immunocytochemical staining has shown that IL-11Rα is expressed by both stromal and epithelial cells, although expression in epithelial cells is greater than that in stromal cells for most parts of the cycle. Expression does not vary much during the menstrual cycle, but there is a slight increase in epithelial IL-11Rα expression during the mid-late secretory phase of the cycle (Cork et al 2002). In addition there appears to be increased expression of IL-11Rα in
stromal cells during decidualisation (Dimitriadis et al., 2000). Expression of gp130 by endometrial epithelial cells in vitro (Cork et al., 2002) and in vivo (Cullinan et al., 1996) and by stromal cells in vitro (Cork et al., 2002) has been reported. Expression is reportedly higher in luminal epithelium than glandular epithelium, but does not vary greatly during the menstrual cycle (Cullinan et al., 1996).

1.10 Leptin

Leptin (or ob protein) is a pleiotropic molecule secreted by white adipocytes that plays a role in the regulation of body weight and food intake (Stephens et al., 1995; Messinis and Milingos, 1999). Leptin suppresses the appetite and increases the metabolic rate (Granowitz, 1997). Recently, it has also been proposed to be involved in the control of reproductive function.

Leptin was discovered in 1994, it was shown that its absence causes obesity in the ob/ob mouse. The leptin receptor was discovered in 1995-1996 and again its absence also causes obesity in the diabetic-obese (db/db) mouse. The name leptin was derived from the Greek root leptos, meaning thin. The original function assigned to leptin was to limit weight gain by reducing food intake as its concentration in blood rises with increasing adiposity.

Animal studies have shown that ob/ob and db/db mice are infertile, stunted, hypothyroid and hypothermic. The two types of mutant are phenotypically identical when the mutant genes are expressed on the same genetic background, but are brought about by different mechanisms. Ob/ob mice lack a factor in their blood that suppresses eating, whereas the db/db mice lack the ability to suppress their food intake in response to this factor. In 1995 leptin was identified as a member of the cytokine family that was missing from the blood of the ob/ob mouse. Leptin is a small peptide product of the ob gene, it is a 16 kDa non-glycosylated polypeptide of 146 amino acids. The precursor form of leptin contains 167 amino acids, and is activated by cleavage of a 21 amino acid residue (Zhang et al., 1994; Ogawa et al., 1995).
Leptin is a hormone that is synthesized and secreted by adipose tissues in proportion to their mass. It provides a signal to the brain of the size of body fat reserves. Leptin acts as an afferent satiety hormone, regulating appetite, weight gain and fat deposition (Zhang et al., 1994; Campfield et al., 1995; Halaas et al., 1995) via a negative feed back loop involving receptors in the hypothalamus (Campfield et al., 1995; Tartaglia et al., 1995). A loss of body fat normally leads to a decrease in leptin secretion that, in turn, promotes food intake in excess of energy expenditure to restore body fat mass. Also, an excessive adipose tissue mass leads to increased leptin secretion, decreased food intake and normalization of body fat mass.

In the absence of functional leptin (as seen in ob/ob mice) animals fail to restrain their food intake and become obese (Harris et al., 1998). The role of leptin in human obesity appears slightly different. Many obese humans do not have circulating leptin levels that are lower than normal, but appear to be refractory to leptin treatment, suggesting that in these individuals, obesity results in a state of leptin resistance (Maffei et al., 1995b). In mice, the roles for leptin include regulation of several hypothalamic-pituitary-endocrine organ axes (adrenal, thyroid, pancreatic islets, gonadal, growth hormone) as well as regulation of thermo-regulation and of feeding. Only two of these roles, regulation of the hypothalamic-pituitary-gonadal axis and regulation of feeding, appear to be of major importance in humans.

In humans serum leptin concentrations correlate with adiposity measured either as body mass index (BMI) or as percentage body fat (Considine et al., 1996). Leptin levels are increased in human obesity, due to a relative insensitivity of the congate hypothalamic receptor and or a defect in the transport of leptin across the blood - brain barrier (Hamilton et al., 1995; Lonnavist et al., 1995; Caro et al., 1996; Considine et al., 1996). The effects of leptin on food intake and energy expenditure are thought to be mediated centrally via neurotransmitters, e.g. neuropeptide Y (Houseknecht et al., 1998).
1.10.1 Leptin Receptor

The leptin receptor is the product of the LEPR gene and belongs to the class I cytokine super family of receptors. The full-length receptor has the signalling capabilities similar to that of the IL-6 type receptor and its helical structure is similar to this cytokine (Baumann et al., 1996; Tartaglia, 1997). Leptin receptor mRNA is expressed in the anterior pituitary, in several areas of the brain and in other tissues (Finn et al., 1998). Leptin receptor mRNA has also been detected in granulosa and thecal cells of the ovary (Zachow and Magoffin, 1997).

The cloned leptin receptor contains two homologous segments representing potential ligand-binding sites. These domains localize to amino acid residues 323-640 and between amino acid residues 428-635 and have been identified as a fibronectin type 3 domain (Fong et al., 1998). In humans and rodents, two major forms of leptin receptors (OB-R) are expressed. The short form (OB-RS) is detected in many organs and is considered to lack signalling capability (Wang et al., 1997) as it has a truncated intracellular domain (Campfield et al., 1996). The long form (OB-RL) with the complete intracellular domain, predominates in the hypothalamus, but is expressed in low amounts in peripheral tissues. OB-RL transduces an intracellular signal by activation of STAT proteins (Takeda et al., 1998). It is thought that the pathways activated by OB-RL are similar to those activated by the G-CSF receptor (G-CSFR), the leukaemia inhibitory factor receptor (LIFR) and other cytokines using glycoprotein, gp130. This is thought to involve the activation of JakS and STAT1, STAT3 and STAT5 (Baumann et al., 1996). There is also some evidence of activation of signalling pathways from the short form of the leptin receptor. It is thought that the short form activates the MAP-kinases pathway (Quinton, 2000) (Figure1.12).
Figure 1.12 Schematic diagram of leptin receptor signalling pathways. After leptin binding and receptor homodimerisation, the long form of the leptin receptor activates the Jak/STAT pathways, while the short form of the receptor activates the MAPK pathways. Taken from Quinton (2000).
1.10.2 The GLN223ARG leptin receptor polymorphism

Single nucleotide polymorphisms (SNP) are the most common genetic variants. Several such polymorphisms exist in the leptin receptor gene including these at codons 109, 204, 223, 343, 492, 656, 976 and 1019 (Figure 1.13). Considine et al. (1996) were the first to identify a single nucleotide polymorphism in the coding region of the leptin receptor gene, at codon 223 (GLN223ARG). This polymorphism results in the substitution of a glutamine with an arginine in the extracellular region of the leptin receptor.

The GLN223ARG polymorphism is associated with an increase in circulating leptin levels, but not with body mass index (BMI) in non-diabetic Pima Indians (Thompson et al., 1997). Homozygosity for the G allele, of this polymorphism is associated with lower plasma leptin levels after correction for obesity and sex. Recently, Chagnon et al. (2000) have identified associations between polymorphisms in the leptin receptor gene and adiposity in middle-aged male subjects. In their study, carriers of the A allele of the GLN223ARG polymorphism, had higher BMI, fat mass, percent fat mass and leptin than non-carriers. They postulate that the leptin receptor gene variation has a significant effect on adiposity, at least in Caucasian male subjects aged 44-64 years. In addition, Quinton et al. (1998) have reported an association of GLN223ARG polymorphism in the leptin receptor gene with serum leptin levels and BMI in postmenopausal Caucasian women. In particular they showed an association between homozygosity of the G allele and leptin levels, fat mass and BMI in postmenopausal women.

1.10.3 Leptin and reproductive system

Leptin is also known to play an important role in reproduction. Mice that are deficient in leptin (ob/ob mice) are infertile, but fertility can be restored by injection of recombinant leptin (Chehab et al., 1996; Cunningham et al., 1999). The amount of body fat stored is known to influence fertility, indicating a link between adipose tissue and the reproductive system (Frisch, 1990).
Figure 1.13 Schematic diagram of the position of leptin receptor polymorphisms, ECD - extracellular domain, TM - transmembrane domain, ICD - intracellular domain (Taken from Quinton thesis, 2000).
Leptin is thought to be a peripheral signal indicating the adequacy of nutritional status for reproductive function (Tataranni et al., 1997), and that low leptin concentrations indicate a status of inadequate nutritional stores to prevent pregnancy that demands additional energy to support a growing fetus (Korbonits et al., 1997). In starved mice, the lack of reproductive function coincides with a fall in plasma leptin concentrations and several neuroendocrine changes. Exogenous leptin injections to these mice restore fertility (Ahima et al., 1997). In addition, leptin infusions are able to restore ovulatory function in an animal model of starvation.

Several studies have suggested that this effect of leptin on reproduction is due to its interaction with the hypothalamic-pituitary-gonad axis and its ability to modulate LH and follicle stimulating hormone (FSH) production (Barash et al., 1997; Yu et al., 1997). In humans, plasma leptin concentrations are higher in females than males (Hickey et al., 1996) and are significantly higher during the luteal phase of the menstrual cycle (Hardie et al., 1997; Quinton et al., 1999a). Other studies, both in the mouse and humans, have suggested that leptin may also have a direct effect on the reproductive organs. The presence of leptin receptors in ovarian granulosa and thecal cells has been shown, and leptin decreases steroid production by these cells (Karlsson et al., 1997; Spicer and Francisco, 1997). Recently, the presence of the leptin receptor and possibly leptin itself by the endometrium has been reported (Alfer et al., 2000; Gonzalez et al., 2000; Quinton et al., 2003).

1.10.4 Leptin during pregnancy
Leptin levels are increased throughout pregnancy in humans, mice and rats, suggesting a significant role for leptin during pregnancy. This increase in circulating leptin concentration may be due to either an increased production by the adipose tissue, the addition of placenta leptin production or an increased level of binding proteins present in the circulation causing an increase in leptin production. Leptin concentrations are higher in pregnant women compared with non-pregnant women. Leptin increases during the first and second trimester to maximal values at about week 28 of pregnancy. They then stabilise in the third trimester and fall to below pre-pregnancy concentrations around the time of delivery (Hardie et al., 1997; Anim-Nyame et al., 2000).
During pregnancy, alterations in fat metabolism occur and the high leptin levels are thought to play a key role in this process. The first two trimesters of gestation, when leptin levels are at their highest, are anabolic as fat stores become deposited. This is essential because, after birth, carbohydrate stores become exhausted and so fat stores serve as the main source of energy. Even though leptin levels are high during this anabolic state there is no decrease in food intake or a decline in metabolic efficiency as might be expected, which indicates a possible state of leptin resistance similar to that which is thought to occur in obesity. A possible explanation for this leptin resistant state may be that soluble leptin receptors (OB-R) are binding with high affinity to leptin, preventing leptin from binding to the signalling form of the leptin receptor (Liu et al., 1997). This leptin resistant state may be crucial in allowing the provision of additional energy stores in preparation for the stress of birth.

The last trimester of pregnancy is catabolic because there is increased lipolysis, elevated serum/plasma concentration of free fatty acids, decreased fat deposition and a significant increase in triglyceride concentrations, probably as a part of physiological preparation for lactation (Hardie et al., 1997; Anim-Nyame et al., 2000). Leptin concentrations are higher in lactating than non-lactating mothers, suggesting that one of the main roles of maternal leptin during pregnancy is to mobilise energy stores in order to prepare for the demand of lactation (Anim-Nyame et al., 2000).

Another possible role of leptin during pregnancy is the regulation of placental growth. Leptin receptors have been found to be expressed in the placenta, both early (7-14 weeks) in gestation and at term (Henson et al., 1998). Leptin concentrations in the umbilical cord blood at birth have been found to correlate with placental size (Schubring et al., 1996). The mechanism by which leptin controls placental growth is unclear, but, evidence suggests that leptin may stimulate placental angiogenesis as shown by experiments carried out on human umbilical venous endothelial cells (Bouloumie et al., 1998).
In contrast to leptin levels, levels of LBA in plasma of pregnant women obtained during weeks 20-30 of gestation were similar to those of non-pregnant women (Lewandowski et al., 1999). These studies suggest that LBA levels do not change during pregnancy and that leptin itself is the more important factor.

1.11 Aim of the project

The aims of this project were to investigate the roles of pro-inflammatory cytokines in recurrent miscarriage by:

1. investigating the distribution of the GLN223ARG polymorphism’s of the leptin receptor gene in women with recurrent miscarriage and comparing this with that already known for control population

2. investigating the distribution of polymorphism’s of the IL1RN and IL1B genes in women with recurrent miscarriage and comparing this with that already known for control population.

3. comparing the presence of each isoform of each gene in women with different causes of miscarriage.

4. comparing expression of endometrial IL-11 and IL-11Rα mRNA and protein in normal fertile women and recurrent miscarriage women during the peri-implantation period.
Chapter 2
Materials and Methods

2.1. Materials & equipments

2.1.1 Genomic DNA extraction

2.1.1.1 Materials
-Tris-base, sucrose, MgCl₂, EDTA, NaCl₂, lauryl sulphate (SDS), HCL, Triton, Sodium perchlorate (Sigma, UK).
-Ethanol (Sigma, UK)
-Chloroform (Sigma, UK)
-isopropanol (2-propanol) (Sigma, UK)
-DNA isolation kit (Gentra Systems, Minneapolis, MN 55441 USA)

2.1.1.2 Equipment
-Sorvall RT 6000D centrifuge (Du Pont Ltd, UK)
-Sorvall super T21 centrifuge (Du Pont Ltd, UK)
-Gene Quant spectrophotometer

2.1.2 Polymerase chain reaction (PCR)

2.1.2.1 Primers
-IL1RN and GLN223ARG leptin receptor primers (Department of Molecular and Genetic Medicine, Hallamshire Hospital, Sheffield)
-IL1B primers (Sigma-Genosys Ltd, Cambridgeshire, U.K.)

2.1.2.2 Materials
-PCR buffer (Bioline Ltd, London, U.K.)
-DNTPs (Bioline Ltd, London, U.K.)
-BIOTAQT™ DNA polymerase (Bioline Ltd, London, U.K.)
-MgCl₂(Bioline Ltd, London, U.K.)
-Mineral oil (Sigma, UK)
2.1.2.3 Restriction endonucleases
Ava I and Msp I enzymes (Promega, Southampton, UK)

2.1.2.4 Equipment
- Thermocycler (Biometra)
- Water bath (Bio-Rad laboratories Ltd)

2.1.3 RNA extraction
2.1.3.1 Materials
- Tri Reagent (Sigma, UK)
- Phosphate buffered saline (PBS) (Sigma)
- Ice-cold chloroform (Sigma, UK)
- Ice-cold isopropanol (Sigma, UK)
- Ethanol (Sigma, UK)

2.1.3.2 Equipment
- Sorvall super T21 centrifuge (Du Pont Ltd, UK)
- Gene Quant spectrophotometer

2.1.4 Reverse transcription-PCR (RT-PCR)
2.1.4.1 Primers
- 7B6 primers (Department of Molecular and Genetic Medicine, Hallamshire Hospital, Sheffield)
- IL-11Ra, IL-11 and IL-11Ra, IL-11 nested PCR and GAPDH primers (Sigma-Genosys Ltd, Cambridgeshire, U.K.)

2.1.4.2 Materials
- RNA later (Ambion, Hinlinden, Cambridgeshire)
- AMV RT buffer and Oligo dT (Invitrogen Life Technologies, USA)
- DNTPs (Bioline Ltd, London, U.K.)
- Mineral oil (Sigma)
- PCR buffer (Bioline Ltd, London, U.K.)
- BIOTAQ™ DNA polymerase (Bioline Ltd, London, U.K.)
- MgCl₂ (Bioline Ltd, London, U.K.)
2.1.4.3 Equipment
Thermocycler (Biometra)

2.1.5 Electrophoresis
2.1.5.1 Agarose gel materials
- Agarose (Sigma, UK)
- Tris borate EDTA buffer (TBA) (Sigma)
- Ethidium bromide (Sigma)
- Loading dye, 1% bromophenol blue (Sigma)
- φX174 DNA/HaeIII marker (Promega, Southampton, UK.)

2.1.5.2 Agarose gel equipment
- Agarose gel plate, comb and tank (Flowgen, model MHU 1510B, Staffordshire)
- Bio-Rad power pack (model 200/2.0)
- Ultraviolet trans-illuminator (BDH 230v 50Hz, TFX-20M)
- Digital camera connected to computer (Kodak electrophoresis documentation and analysis system, Rochester, NY)

2.1.5.3 Polyacrylamide gel materials
- Acrylamide/bis-acrylamide (Sigma)
- Tris borate EDTA (TBA) buffer (Sigma)
- TEMED (N,N,N,N-Tetramethyl-Ethylendiamine) (Sigma)
- Ammonium persulphate (Sigma)
- Loading dye, bromophenol blue (Sigma)
- φX174 DNA/HaeIII marker (Promega, Southampton, UK.)
- Ethidium bromide (Sigma)

2.1.5.4 Equipment for polyacrylamide gel
- Polyacrylamide gel apparatus (Bio-Rad, Hertfordshire)
- Ultraviolet trans-illuminator (BDH 230v 50Hz, TFX-20M)
- Digital camera connected to a computer (Kodak electrophoresis documentation and analysis system, Rochester, NY)
2.1.6 Measuring plasma IL-1β concentration

2.1.6.1 Materials
Quantikine immunoassay kit (R&D Systems, Abingdon, UK.)

2.1.6.2 Equipment
Wallac 1420 multilable counter (Wallac, Finland)

2.1.7 Preparation of PCR product DNA for sequencing

2.1.7.1 Materials
-DNA isolation QIAquick gel extraction kit (QIAGEN Ltd, West Sussex, UK)
-Agarose gel containing PCR product
-Tris acetate EDTA buffer (TAE) (Sigma)
-Loading dye, bromophenol blue (Sigma)
-ΦX174 DNA/HaeIII marker (Promega, Southampton, UK)
-Ethidium bromide (Sigma)
-Isopropanol (2-propanol) (Sigma, UK)

2.1.7.2 Equipment
-Thermocycler (Biometra)
-Water bath (Bio-Rad laboratories Ltd)
-Sorvall super T21 centrifuge (Du Pont Ltd, UK)

2.1.8 Immunocytochemistry

2.1.8.1 Materials

2.1.8.1.1 Cryostat materials
-Cryo-M-Bed embedding compound (Bright Instrument Company Ltd, Huntington, UK)
-3.7% paraformaldehyde in PBS (Sigma)
-Phosphate-buffered saline PBS (Sigma)
-Ice-cold methanol (Sigma)
-Ice-cold acetone (Sigma)
2.1.8.1.2 Immunocytochemistry materials

- VECTASTAIN ABC kit and DAB kit (Vector Laboratories, Inc., Burlingame, USA)
- Phosphate-buffered saline PBS (Sigma)
- Haematoxylin (Vector, UK)
- Ethanol (BDH, Poole, UK)
- Xylene and DePeX (BDH, Poole, UK)
- IL-11Ra, IL-11 primary antibodies (R&D Systems, Abingdon, UK.)

2.1.8.2 Equipment

Leica cryostat at -20°C
2.2 Methods

2.2.1 Human subjects
All blood samples and endometrial biopsies were obtained from the Jessop Hospital for Women in Sheffield. All samples were collected with patients' consent. Ethical committee approval had been obtained for all aspects of these study. All of the women were aged between 20 and 40, had menstrual cycles of between 25 and 35 days and all were of European Caucasian origin.

2.2.1.1 Peripheral blood
Peripheral blood was obtained from 206 women who were attending the recurrent miscarriage clinic, and had suffered at least three miscarriages. The women were investigated according to an established protocol (Li et al., 1998) to establish the cause of miscarriage. These tests included: analysis of parental chromosomes; presence of coagulation defects (antiphospholipid antibodies including anti-lupus coagulant and defects in clotting proteins); presence of PCO; endometrial development; thyroid function tests and presence of a uterine structural abnormality. If all the tests were negative, the cause was termed unexplained. Some women did not complete all the investigations, in this case the cause is defined as unknown. 86 of these women had unexplained recurrent miscarriage, 4 had abnormal parental karyotype, 41 had coagulation disorders, 10 had PCO, 27 had retarded endometrium, 16 had abnormal thyroid function and 31 had uterine structural abnormalities. In 27 women, 2 causes were identified and 2 women had three different causes. In 25 women the cause was unknown.
2.2.1.2 Endometrial biopsies

2.2.1.2.1 Normal fertile women
Endometrial biopsies were obtained from women undergoing surgery for non-endometrial pathology. All these subjects had normal ovulation, normal uterine anatomy, had no steroid treatment for at least 2 months prior to the study and were all of proven fertility. The day of the cycle in which the biopsies were taken was calculated from the date of the last menstrual period.

2.2.1.2.2 Women with recurrent miscarriage
Precisely timed endometrial biopsies were obtained from women attending the recurrent miscarriage clinic. They all had suffered at least three miscarriages. The day in the cycle that the biopsies were obtained was calculated from the day of the LH surge and all biopsies were obtained between days LH+7 and LH+10.

2.3 Collection of blood
10 ml of peripheral blood was collected into tubes containing EDTA for anticoagulation, immediately snap frozen in liquid nitrogen and stored at -70°C. Plasma was prepared by centrifugation at 1000 g from a further 10 ml blood sample obtained from the same women and stored at -70°C.

2.4 Collection of biopsies
Endometrial biopsy samples were collected directly from the operating theatre (controls) or outpatient's clinic (recurrent miscarriage women) using a pipelle sampler. If the biopsy was large enough it was divided into 2 parts. 1 part was placed into “RNA later” to prevent mRNA breakdown and used for RT-PCR analysis, while the other was kept dry for immunocytochemical analysis. Both samples were immediately snap frozen in liquid nitrogen and stored until required.
2.5 Genomic DNA extraction

2.5.1 DNA extraction by the standard methods

For the first 102 blood samples, DNA was extracted by standard methods used in our laboratories. Blood was placed into a 50 ml polypropylene centrifuge tube (Falcon) and made up to 50 ml with lysis buffer A (Tris base 0.01M, sucrose 0.32M, MgCl₂ 0.15M, in H₂O) adjusted to pH 8 with concentrated HCl. The buffer was then autoclaved and once cool, 1% Triton x-100 was added. Buffer A was added to induce hypotonic lysis of the cells and, thus, remove the haemoglobin and other cellular proteins. The tubes were mixed end-over-end for 4 minutes at room temperature and then centrifuged (Sorvall RT 6000D) at RT, 1000g for 15 minutes. The supernatant was removed and the pellet (containing the white cell nuclei) resuspended in 20 ml of buffer A. The tubes were then centrifuged again at room temperature at 1000g for 15 minutes. The supernatant was discarded and 1 ml of extraction buffer B was added (Tris base 0.4M, EDTA 0.06M, NaCl₂ 0.005M. in H₂O) adjusted to pH 8 with concentrated HCl. The buffer was autoclaved and, once cool, made-up to 1L; sodium lauryl sulphate SDS was then added to a final concentration of 1%. Buffer B was added to resuspend the pellet. The resuspended pellet was transferred to an Eppendorf tube, 300μl of 5M sodium perchlorate (to precipitate the proteins) was added and mixed end-over-end for 10 minutes. The tube was subjected to high speed microcentrifugation, (10,000g) for 10 minutes, after which 600μl of supernatant was added to a clean Eppendorf tube and 700μl of ice-cold chloroform added. This procedure removes the protein and leaves DNA in the supernatant. After mixing end-over-end for 3 minutes the tubes were centrifuged for 10 minutes at full speed (10,000g). The top layer was transferred to a clean Eppendorf tube and twice the volume of ice-cold ethanol added. The ethanol precipitates the DNA. After mixing gently the tube was centrifuged (Sorvall Super T21) at 4°C, at 10,000g for 15 minutes. The supernatant was removed and the DNA pellet air-dried for 10
minutes. The pellet was then resuspended in 200μl sterile distilled water and mixed end-over-end at 4°C overnight.

2.5.2 DNA extraction by using a DNA isolation Kit
For the second 104 blood samples, a DNA isolation kit was used (Gentra Systems, Minneapolis, MN 55441 USA). 3 ml of whole blood was added to a 15 ml polypropylene centrifuge tube (Falcon) containing 9 ml RBC lysis solution. The tube was inverted to mix and incubated for 10 minutes at room temperature. The tube was then centrifuged at 2,000 g for 10 minutes. The supernatant was removed leaving behind the white cell pellet and about 100μl of residual liquid. The tube was then vortexed vigorously to resuspend the cells in the residual liquid. 3 ml of cell lysis solution was added and the cells were lysed by pipetting up and down. 15μl RNase solution was added to the cell lysate and the tube was mixed by inverting 25 times, followed by incubation at 37°C for 15-60 minutes. After cooling to room temperature, 1 ml protein precipitation solution was added and the tube was vortexed for 20 seconds to mix the protein precipitation solution uniformly with the cell lysate. The tube was then centrifuged at 2,000 g for 10 minutes. The supernatant containing the DNA was poured into a clean 15 ml polypropylene centrifuge tube (Falcon) containing 3 ml 100% isopropanol (2-propanol). The tube was mixed by inverting gently 50 times until the white threads of DNA formed a visible clump. After centrifugation at 2,000 g for 3 minutes, the DNA was visible as a small white pellet. The supernatant was discarded and the tube drained briefly on clean absorbant paper. Ethanol (3ml) was then added and the tube inverted several times to wash the DNA pellet. The tube was centrifuged at 2,000 g for 1 minute and then the ethanol carefully removed. The tube was inverted and drained on clean absorbent paper and allowed to air dry for 10-15 minutes. The DNA was hydrated by addition of 250 μl DNA hydration solution and incubation at 65°C for 1 hour and then overnight at room temperature.
2.5.3 Analysis of DNA purity and concentration
The concentration of genomic DNA was estimated by measurement of its absorbance at 260nm (GeneQuant spectrophotometer). In a 1 cm-path length quartz cuvette, 1 absorbance unit at 260nm is equal to 50mg/ml of double-stranded DNA. DNA samples were diluted (1:1000 in water) and the absorbance measured at 260 and 280nm. The ratio of optical densities gives an indication of the purity of the DNA and should be between 1.7-2.1 (1.8 for pure DNA). The following equation was then used to calculate the DNA concentration:

\[
\text{DNA concentration (ng/μl)} = \left( \frac{\text{optical density at 260nm}}{0.02 \text{ (dilution factor)}} \right) \times 100
\]

The stock DNA was diluted in water to a final concentration of 50ng/μl. Samples were stored at -70°C until required.

2.6 Polymerase chain reaction analysis (PCR)
PCR is a simple technique, where a short specific region of a DNA molecule, a single gene for instance, is copied many times by a DNA polymerase enzyme. Taq polymerase is used, which, unlike most DNA polymerases, is not inactivated by heat treatment. Primers, that bind only to the nucleotide sequence flanking the DNA region of interest are used to initiate replication. To begin a PCR amplification, the enzyme is added to the primed DNA template and incubated so that it synthesises new complementary strands. The mixture is then heated to 96°C so that the newly synthesized strands detach from the template. The mixture is then cooled, enabling more primers to hybridize at their respective positions, including positions on the newly-synthesised strands. A second round of DNA synthesis now proceeds, the cycle of denaturation-hybridization-synthesis is repeated, usually 25-35 times, resulting in the synthesis of several hundred million copies of the amplified DNA fragment.
To enable specific amplification of cDNA, two oligonucleotide primer sequences are designed that, when added to cDNA will bind specifically to complementary DNA sequences immediately flanking the desired target region.

2.6.1 Polymerase chain reaction (PCR) analysis for GLN223ARG Leptin receptor polymorphism

The leptin receptor polymorphism GLN223ARG is in exon 4 of the leptin receptor gene (Echwald et al., 1997) and is contained in a 416 bp PCR product produced when genomic DNA is amplified by the following primer sequences:

Forward primer 5' TCC TCT TTA AAA GCC TAT CCA GTA TTT 3'
Reverse primer 5' AGC TAC CAA ATA TTT TTG TAA GCA TA 3'

PCR reactions were carried out in a 50μl volume containing: PCR buffer (a final concentration of 20mM Tris-HCl, 50mM KCl), 2.5mM MgCl₂, 0.6μM of the forward primer, 0.6μM of the reverse primer, 0.2mM of dNTPs, 2.5 units of Taq DNA polymerase and 100ng of DNA template in sterile water. In this and all other PCR reactions a negative control was included in each PCR reaction, where DNA was replaced with 2μl of water. The PCR mixtures were always overlaid with 40μl of mineral oil to prevent evaporation during the thermal cycle stages. The tubes in this and all other PCR reactions were placed onto a thermocycler (Biometra). The thermal cycle was as follows: heating to 95°C for 3 minutes, 35 cycles of heating to 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute, followed by a final extension period of 72°C for 5 minutes and annealing completion of 55°C for 5 minutes.
2.6.2 Genotyping GLN223ARG by restriction digest with Msp I

Digestion of the PCR product with the restriction enzyme Msp I allows detection of the alleles of GLN223ARG. Msp I cuts at sequences:

\[ \begin{align*}
&C \downarrow \text{CG G} \\
G &\text{GC} \downarrow \text{C}
\end{align*} \]

The PCR products containing the nucleotide sequence coding for the amino acid arginine (GCC) are cut to produce two smaller products (251bp and 165bp), while the product containing the nucleotide sequence for glutamine is not cut. The digestion reaction was carried out in a total volume of 30μl, containing 20μl of PCR product, 3μl of buffer (a final concentrations of 6 mM Tris-HCl, 50 mM NaCl, 6 mM MgCl₂, and 1 mM DTT), 6 μl of water and 1μl (10 units) of Msp I enzyme. The tubes were incubated at 37°C for 2 hours. After digestion, the samples were separated by gel electrophoresis using a 2% agarose gel in order to visualise the different products.

2.6.3 Polymerase chain reaction (PCR) analysis for Interleukin-1 receptor antagonist IL1RN polymorphism

The intron 2 variable length polymorphism in the IL1RN gene was amplified using the following primer sequences, which have been published previously (Lennard et al. 1992). The sequences for the primers used were as follows:

Forward primer 5’ CTC AGC AAC ACT CCT AT 3’ primer 139
Reverse primer 5’ TCC TGG TCT GCA GGT AA 3’ primer 157

PCR reactions were carried out in a 50μl volume containing: PCR buffer (a final concentration of 20mM Tris-HCl, 50mM KCl), 1.75mM MgCl₂, 1μM of the forward primer, 1μM of the reverse primer, 0.2mM deoxynucleotide triphosphates (dNTPs), 100ng of DNA template and 1.25 units of Taq DNA polymerase (recombinant from *Thermus aquaticus*) in sterile water. The thermal cycle was as follows: heating to 96°C for 1 minute, 35 cycles of heating to 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 2 minutes, followed by a final extension period of 72°C for 5 minutes and
annealing completion of 55°C for 5 minutes. The PCR products were stored at 4°C until used for electrophoresis. The PCR product size varies depending on the number of repeat regions: for 2 repeats it is 240bp, for 4 repeats it is 410bp, for 5 repeats it is 500bp.

2.6.4 Polymerase chain reaction analysis for IL1B - 511 C/T polymorphism

The region of the IL1B gene containing the -511 C/T sequence polymorphism was amplified using the following primers sequences (di Giovine, et al. 1992). This produces a 304 bp PCR product which contains the C/T polymorphic site. The primer sequences used were the following:

Forward primer 5′-TGG CAT TGA TCT GGT TCA TC-3′ primer
Reverse primer 5′-GTT TAG GAA TCT TCC CAC TT-3′ primer
INV20

PCR reactions were carried out in a 50µl volume containing: PCR buffer (a final concentration of 20mM Tris-HCl, 50mM KCl), 2.5mM MgCl₂, 2µM of the forward primer, 2µM of the reverse primer, 0.2mM of the dNTPs, 1.25 units of Taq DNA polymerase and 100ng of DNA template in sterile water. DNA was amplified as follows: 2 cycles of heating to 95°C for 2 minutes, annealing at 53°C for 1 minute and extension at 74°C for 1 minute, followed by 35 cycles of heating to 95°C for 1 minute, annealing at 53°C for 1 minute and extension at 74°C for 1 minute then a final 3 cycles of heating to 94°C for 1 minute, annealing at 53°C for 1 minute and extension at 74°C for 5 minutes. The PCR products were stored at 4°C until used for electrophorsis and restriction endonuclease digestion.
2.6.5 Genotyping IL1B -511 C/T by restriction digest with Ava I enzyme

Digestion of the PCR product with the restriction enzyme Ava I allow detection of the C allele of IL1B-511 C/T. The Ava I cutting site is: 5'‐CC↓CGAG-3', the products containing the C base are cut to produce two smaller products (190bp, 114bp), whereas the sequences containing the T base are not cut. The digestion reaction was carried out in a total volume of 33.4μl, containing 30μl of PCR product, 3μl of the buffer B (10x buffer), and 0.4μl (0.1mg/ml) of Ava I enzyme. The tubes were incubated at 37°C overnight. After digestion, the samples were separated by gel electrophoresis using a 6% polyacrylamide gel in order to visualise the different products.

2.7 RNA extraction from cultured cells, lymphocytes and tissue

Extraction of RNA from cultured cells, lymphocytes and tissue used a modified method first described by Chomczynski and Sacchi (1987). Homogenisation or lysis of the cells or tissue in Tri Reagent (Sigma) allows total RNA to be retained whilst the cells are disrupted and the cell components are removed.

2.7.1 Extraction of RNA from cultured cells

mRNA extracted from cultured endometrial epithelial cells was used to develop the RT-PCR methods. The cell culture media was removed and the cells washed twice in 10ml of 1x phosphate buffered saline (PBS) to remove any cell debris. 4ml of Tri Reagent was added to the flask. The liquid was pipetted vigorously until the cells had lysed. The lysed cells were transferred to four 1.5ml Eppendorf tubes and stored frozen at -80°C until the RNA extraction procedure was carried out.
2.7.2 Extraction of RNA from tissue samples

The endometrial biopsies were small pieces of tissue samples, approximately 1g in weight. They had been snap frozen in liquid N₂. Samples from liquid nitrogen were transferred to a mortar and 1ml of Tri Reagent was added. The frozen samples were ground using a sterile pestle and the mortar until completely broken down. The tissue was kept frozen (1) to stop the RNA from degrading and (2) to allow the samples to be easily fragmented. After that, the tissue was further broken up by passing through a wide base needle attached to a syringe until a suspension was produced. This was transferred to a clean Eppendorf tube and stored frozen at -80°C until required.

2.7.3 RNA extraction protocol

Frozen tissue and cell samples were allowed to thaw on ice. 100μl of ice-cold chloroform (per ml of Tri Reagent) was added and the tubes were incubated on ice for 10 minutes. The samples were then centrifuged (Sorvall Super T21) for 15 minutes at 10,000g at 4°C. The upper aqueous layer (approximately 500μl) was removed and an equal amount of ice-cold isopropanol was added. This was inverted gently and allowed to incubate on ice for 1 hour, this process allowed precipitation of the RNA. The sample was then centrifuged, as before, for 15 minutes. The supernatant was discarded and the pellet gently resuspended in 250μl of 80% ethanol. This was centrifuged at 4°C at 10,000g for 5 minutes, the ethanol removed and the pellet allowed to air-dry for 10 minutes. The pellet was then resuspended in 20-50μl of sterile water.

2.7.4 Analysis of RNA purity and concentration

The concentration of RNA in each sample was estimated by measurement of the absorbance at 260nm. In a 1 cm-path length quartz cuvette, 1.0 absorbance unit at 260nm is equal to 40μg/ml of RNA. RNA samples were diluted (1:1000 in water) and the absorbance measured at 260 and 280nm.
The following equation was used to calculate the RNA concentration:

\[ \text{RNA concentration (\mu g/\mu l)} = (\text{optical density at 260nm} \times 200 \times \text{dilution factor}) \times 0.04 \]

The samples were adjusted to a concentration of 1\(\mu g/\mu l\) with sterile water. The purity of the RNA sample was also assessed by measuring the ratio of absorbencies at 260nm and 280nm. A ratio of 2.0 was considered pure. The samples were stored at (-80°C) until required.
2.8 mRNA Expression analysis

2.8.1 Reverse transcriptase-PCR (RT-PCR)

Reverse transcriptase-polymerase chain reaction (RT-PCR) is a two-step process that can be used to identify the presence of messenger RNA (mRNA). The first step uses reverse transcriptase (RT) to make a DNA sequence that is complementary (cDNA) in base sequence to the mRNA. This step is required because the polymerase used in PCR will only amplify DNA. PCR is then used to amplify the defined cDNA produced from the RT step as described in section 5.

The sequences for all of the primers used are as follows:

**7B6**

Forward primer  
-5’ AGC CGT AGA CGG AAC TTC CA 3’-

Reverse primer  
-5’ CTA AAA CAG CGG AAG CGG T 3’-

(Francis and Duff, 1993)

**IL-11Ra**

Forward primer  
-5’ TGC AGC TGG GCT ACC CTC C 3’-

Reverse primer  
-5’ CGG GCT CCA GGT GCT CCA 3’-

(Pflanz et al, 1999)

**IL-11**

Forward primer  
-5’ GAC AGG GAA GGG TTA AAG GC 3’-

Reverse primer  
-5’ AGC TGT AGA GCT CCC AGT GC 3’-


**IL-11Ra** (Nested)

Forward primer  
-5’ GCT ACC TCA CCT CCT ACA 3’-

Reverse primer  
-5’ ATC ACC TCC TCC AGT CCA 3’-

(The primers were designed in-house by Dr. Alex Blakemore)

**IL-11** (Nested)

Forward primer  
-5’ CTC GAG TTT CCC CAG ACC 3’-

Reverse primer  
-5’ CGT CAG CTG GGA ATT TGT 3’-

(The primers were designed in-house by Dr. Alex Blakemore)

**GAPDH**

Forward primer  
-5’ TGA TGA CAT CAA GAA GGT GGT GAA G 3’-

Reverse primer  
-5’ TCC TTG GAG GCC ATG TGA GGC CAT 3’-

(The primers were designed in-house by Dr. Maria Blair)
2.8.2 First strand cDNA synthesis

First strand cDNA synthesis was carried out in 40μl reaction mix containing: 5 x AMV reverse transcriptase buffer (a final concentrations of 100mM Tris-HCl, 10mM MgCl₂, 10mM DTT); 50mM KCl; 1mM dNTPs; 0.6μg of oligo (dT) in sterile water. For each RNA sample, triplicate tubes were prepared, to two tubes 4μl of 1μg/μl total RNA was added. 1.2μl of AMV (avian myeloblastosis virus) reverse transcriptase RT was added to one of these tubes and 1.2μl of pure water was added to the other tube which acted as a control reaction. To the third tube, 4μl of pure water and 1.2μl of RT were added, which acted as a negative control. Each tube was overlaid with 40μl of mineral oil to prevent evaporation during the thermocycler stages. The samples were incubated on the thermocycler (Biometra) at 37.5°C for 1 hour to allow cDNA synthesis, then heated for 5 minutes at 99°C to denature the RT enzyme. The samples were either used directly in PCR or were stored at -20°C until required.

2.8.3 Polymerase chain reaction for 7B6 gene

This initial PCR analysis was carried out using the housekeeping gene 7B6, to determine whether there was any genomic DNA contamination and to ensure that enough mRNA had been obtained. The PCR reaction was carried out in a 20μl volume containing: PCR buffer (final concentrations 20mM Tris-HCl, 50mM KCl); 1.25mM MgCl₂; 0.3μM of the forward primer; 0.3μM of the reverse primer; 1.25 units of Taq DNA polymerase and 5μl of appropriate cDNA sample in sterile water. The thermal cycle used for amplification was: denaturation at 96°C for 5 minutes, 35 cycles of: denaturation at 94°C for 1 minute; primer annealing at 58°C for 1 minute; extension at 72°C for 1 minute; a final extension at 72°C for 5 minutes and annealing completion at 55°C for 5 minutes. The PCR products were stored at 4°C until required for electrophoresis. The PCR product size for cDNA was 434bp.
2.8.4 Polymerase chain reaction for interleukin-11 receptor α (IL-11R α)
The PCR reaction was carried out in a 20μl volume containing: PCR buffer (20mM Tris-HCl, 50mM KCl); 2.5mM MgCl₂; 0.3μM of the forward primer; 0.3μM of the reverse primer; 1.25 units of Taq DNA polymerase and 5μl of cDNA sample in sterile water. PCR was carried out in a thermocycler under the following conditions: denaturation at 96°C for 3 minutes; 35 cycles of heating to 94°C for 30 seconds; primer annealing at 58°C for 45 seconds; extension at 72°C for 1 minute. The final extension was at 72°C for 5 minutes, then the samples were stored at 4°C until required for electrophoresis. The PCR product size for cDNA was 631bp.

2.8.5 Polymerase chain reaction for interleukin-11 (IL-11)
The PCR reaction was carried out in a 20μl volume containing: PCR buffer (20mM Tris-HCl, 50mM KCl); 1.5mM MgCl₂; 0.12μM of the forward primer; 0.12μM of the reverse primer; 1.25 units of Taq DNA polymerase and 5μl of cDNA sample in sterile water. PCR was carried out on a thermocycler under the following conditions: denaturation at 94°C for 5 minutes; 30 cycle of heating to 94°C for 1 minute; primer annealing at 58°C for 1 minute; extension at 72°C for 1 minute. Final extension was at 72°C for 10 minutes, then samples were stored at 4°C until required for electrophoresis. The PCR product size for cDNA was 355bp.

2.8.6 Reverse Transcriptase Polymerase Chain Reaction for GAPDH
RT-PCR for glyceraldehyde phosphate dehydrogenase (GAPDH) was used as internal standard because its expression doesn't change during the menstrual cycle and it has also been used previously as an internal standard in studies on the endometrium (Dimitriadis et al., 2000).
The PCR reaction were carried out in 20μl volume containing: PCR buffer (20mM Tris-HCl, 50mM KCl), 125μM of dNTPs; 2.5mM MgCl₂; 0.4μM of the forward primer; 0.4μM of the reverse primer; 5 units of Taq DNA polymerase and 1μl of cDNA sample in sterile water. The thermal cycle was as follows: denaturation at 95°C for 5 minutes; 30 cycle of heating to 95°C for 1 minute; primer annealing at 58°C for 1 minute; extension at 72°C for 1 minute. Final extension was at 72°C for 10 minutes, then the PCR products were stored at 4°C until required for electrophoresis. The product size for GAPDH was 240bp.

2.8.7 Nested Polymerase Chain Reaction

As it became apparent that the single PCR reaction would not be able to detect IL-11Rα and IL-11 in both endometrial epithelial cells and biopsies, a nested PCR reaction was attempted. Nested polymerase chain reaction is a two-step process that can be used to identify specific amplification of DNA. The first step is an initial RT-PCR that results in amplification of the specific region of DNA of interest. The second step is another PCR used to amplify the region of interest further. To enable this amplification, two oligonucleotide primer sequences were designed which would bind to nucleotide sequences present inside the first primer sequences. These sequences were present on the product of the first PCR. When these primers are added to the PCR product they will bind specifically to the PCR product and allow further amplification of the desired target region (figure 2.1).

2.8.8 Nested Polymerase Chain Reaction for IL-11Rα and IL-11

Primers used in the nested PCR for IL-11Rα and IL-11 were designed in-house by Dr. Alex Blakemore using Oligo™ primer analysis software (version 4.0 n). This software enables the design and analysis of primer sequences at specific points along the DNA sequence of interest and subsequent calculation of the optimum annealing temperature for the PCR reaction.
The PCR reaction mixes were the same as those described previously for IL-11 and IL-11Rα except that the cDNA template was replaced by PCR product. The thermal cycle stages were also similar except that the annealing temperature for IL-11Rα was 56.1°C and for IL-11 was 56.3°C. The PCR products were stored at 4°C until required for electrophoresis. The PCR product size for IL-11Rα was 562bp and for IL-11 was 112bp.
Figure 2.1 Sequence of the IL11 cDNA showing the outer primers in blue colour and nested primers in red colour (taken from NCBI).

(Forward primer) GAC AGG GAA GGG TTA AAG GC ccc ggc tcc
ctg ccc cct gcc ctg ggg aac ccc tgg ccc tgt ggg gac atg aac tgt gtt
tgc cgc ctg gtc ctg gtc gtg ctg agc ctg tgg cca gat aca gct gtc
gcc cct ggg cca cca cct ggc ccccc (Forward primer) CTC GAG
TTT CCC CAG ACC ctc ggg ccg agc tgg aca gca ccg tgc tcc
tga ccc gct ctc tcc tgg cgg aca cgc ggc agc tgg ctg cac agc tga
ggg (Reverse primer) ACA AAT TCC CAG CTG ACG ggg acc
aca acc tgt att ccc tgc cca ccc tgg cca tga gtg cgg gg
(Reverse primer) GC ACT GGG AGC TCT ACA GCT ccc agg tgt
gct gac aag gct gcg agc gga cct act gtc cta cct gcg gca cgt gca
gtg
2.9 Electrophoretic Techniques

2.9.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate the PCR and RT-PCR products on the basis of fragment size. A 2% gel was made by dissolving 1 g agarose in 50 ml 1x Tris borate, EDTA buffer (TBE, pH 8), (89mM Tris, 89mM Boric acid, 2.5mM EDTA in distilled water). 2μl of ethidium bromide (stock solution 10mg/ml in water) was added to the cooling gel to enable visualisation of the bands. The gel mixture was then poured into the gel plate and left to set around a gel comb (Flowgen, Shenstone, Staffordshire). The gel was placed in a tank (Flowgen, model MHU 1510B) containing 1x TBE, and the PCR products added to the wells. Each PCR product (15μl) was mixed with 5μl loading dye (1% bromophenol blue in 10% sucrose in 50ml 1xTBE) and then this mixture added to the appropriate well. A marker (ΦX174DNA/HaeIII) (0.5μl marker in 15μl of water and 5μl of loading dye) was run parallel to PCR products in every gel, and was used to estimate the size of any product seen by comparison with the sizes of marker bands. Electrophoresis was carried out at 90-95 V for 60 minutes. The DNA was then visualised on an ultraviolet trans-illuminator. Images were recorded using a digital camera connected to a computer.

2.9.2 Polyacrylamide gel electrophoresis

The PCR products for the IL1B polymorphism were small and therefore polyacrylamide gel electrophoresis was used to separate them. A 6% acrylamide/bis-acrylamide gel solution was prepared containing: 4ml acrylamide/bis-acrylamide (40% stock solution mixture, Ratio 19:1), 4ml of 5x Tris Borate EDTA (TBE) buffer and 12ml distilled water. 20μl Temed (N,N,N,N-Tetramethyl-Ethylenediamine) and 120μl ammonium persulphate (10%) was then added and the gel solution poured between two glass plates. Temed and APS cause the acrylamide mixture to polymerise forming a polyacrylamide gel matrix. Before the gel had set, a comb with 10 wells was placed in the top of the gel and the gel left to set around it.
The comb was removed, the gels in the glass plates were then placed into a tank containing running buffer (1x TBE). The PCR products were added to the wells (15µl + 5µl loading dye). DNA marker (ΦX174 DNA/HaeIII) (0.5µl + 15µl water + 5µl loading dye) was run parallel to PCR products in every gel. Electrophoresis was carried out at 200V for 40 minutes. The gels were removed from the electrophoresis apparatus and placed into a 0.001% ethidium bromide solution in TBE buffer for 10 minutes. The gels were subsequently destained in water for a few minutes and viewed under ultra-violet light in the same trans-illuminator. Images were recorded using the same digital camera, and computer system used to analysis agarose gels.

2.10 Preparation of PCR product DNA for sequencing
In order to check that the RT-PCR reaction for IL-11Ra had produced the correct product and was, therefore, detecting the right gene product, 3 samples were prepared and sent for automatic sequencing.

2.10.1 Isolation of specific DNA product by using QIAquick gel extraction kit
A 50ml 1% agarose gel was made as before but, using 1x Tris acetate EDTA buffer (TAE) (0.04M Tris-acetate, 1mM EDTA) instead of TBE. The gel was cooled and 2µl of ethidium bromide (stock solution 10mg/ml) was added. A comb with a sample volume of 60µl of DNA was used. A 50µl PCR reaction was carried out to provide a template for sequencing. This total volume was mixed with 10µl of loading dye (20% sucrose solution in 1x TAE containing bromophenol blue) and the total of 60µl was loaded onto the gel. DNA marker (ΦX174 DNA/HaeIII) was also added as before to allow for product sizing. Electrophoresis was carried out for 1 hour at 95V. After 1 hour, the bands on the gel were visualised by UV transillumination. A sterile blade was used to excise the band of interest from the agarose gel.
The gel slice was weighed in a tube, 3 volumes of buffer QG (guanidine thiocyanate) was added to 1 volume of gel (300µl of buffer QG to each 100mg of gel). The gel slice was dissolved by heating at 50°C for 10 minutes. After the gel slice had dissolved completely, the colour of the mixture should be yellow indicating a pH≤7.5. To increase the yield of DNA, isopropanol (100µl/100mg gel slice) was added to the sample and mixed. The sample was applied to the QIAquick column, which was placed into a 2ml collection tube and centrifuged for 1 minute (at ≥10000 x g, 13000 rpm). The liquid in the collection tube was discarded and the QIAquick column was placed back in the same collection tube. To wash, 0.75ml of buffer PE (containing ethanol) from the kit was added to the column and centrifuged for 1 minute. The liquid in the collection tube was discarded and the column was placed back again in the same collection tube and centrifuged for 1 minute in order to removed the residual ethanol from buffer PE. Then the QIAquick column was placed into a clean 1.5ml microcentrifuge tube. To elute DNA, 50µl of elution buffer EB (10mM Tris-HCl, pH 8.5) was added to the centre of the membrane in the column and centrifuged for 1 minute at maximum speed. The sample was then used for automatic sequencing.

2.10.2 Automatic sequencing
The 3 samples were sent to MWG-BIOTECH UK LTD for sequencing. Forward and reverse primers (at a concentration of 15pM) were supplied with the DNA. The primers were the same as those used in the PCR reaction. Sequence results could only be obtained for two of the three products. A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi) was carried out on about 150 bp of good sequence from the two products which did yield results.
2.11 Analysis of expression of IL-11Rα, IL-11 mRNA compared to GAPDH using Kodak Digital Science™ ID Image Analysis Software

RT-PCR and nested RT-PCR for IL-11Rα and IL-11 mRNA was carried out as described in section (2.9.1). The products from these reactions and those for GAPDH were run together in each well of an agarose gel. The gel images produced were analysed using Kodak Digital Science™ ID Image Analysis Software. This software programme analysed the bands in the gel according to the marker (ΦX174 DNA/Hae III) and measured the molecular weight, intensity, mean intensity and mass weight for each band in the gel. Bands corresponding to IL-11, IL-11Rα and GAPDH were identified, from their size and the intensity measurements corresponding to their band size obtained. An intensity ratio of IL-11 band size : GAPDH band size and IL-11Rα band size : GAPDH for each of the biopsies was calculated.

2.12 Plasma IL-1β concentration

The level of IL-1β in the plasma of blood samples taken from the RM women was measured using a Quantikine immunoassay kit. A 96-well plate (Quantikine immunoassay kit) was coated with a mouse monoclonal antibody against IL-1β. 100μl of assay diluent was added to each well, followed by 150μl of standard (recombinant human IL-1β in a buffered solution) or plasma sample. The plate was incubated for 14-20 hours at room temperature. The wells were then washed with wash buffer before adding 200μl of IL-1β conjugate (poly-clonal antibody against IL-1β, conjugated to alkaline phosphatase) to the wells. Then the plate was incubated for 3 hours at room temperature. Following another wash, 50μl of substrate solution was added to each well, and the plate incubated for 45 minutes at room temperature. 50μl of amplifier solution was then added to each well, followed by incubation for 45 minutes at room temperature. After the incubation, 50μl of stop solution was added. The absorbance was measured at 490nm using a Wallac 1420 multilabel spectrophotometer. The intra-assay and inter-assay
coefficients variation, both were less than 10%. The sensitivity of the assay was less than 0.1 pg/ml.

2.13 Linkage disequilibrium analysis
Linkage disequilibrium occurs when a combination of alleles of a set of genetic markers (a haplotype), occurs significantly more or less frequently in a population than would be expected. If linkage disequilibrium exists between alleles of two polymorphisms then one might be acting as a marker for the presence of the another, nearby polymorphism. There are several measures of linkage disequilibrium. The 2LD computer programme was used to calculate D’ (a measure of the strength of LD). The 2LD programme which is used to assess significance as described above, also gives a $\chi^2$ value (Zhao JH, 2002).

2.14 Haplotype analysis
The IL1B and IL1RN genes are present on the same chromosome (chromosome 2). If markers lie very close together on a chromosome, sets of alleles on the same small chromosome segment tend to be transmitted as a block through pedigrees and through populations. These block of alleles are known as haplotypes. The EH programme (Terwilliger J, Ott J 1994) was used to analyse the frequency of IL1B and IL1RN haplotypes in each population of women (RM women & controls, primary & secondary miscarriage, PCOS-RM & RM without PCOS groups).

2.15 Immunocytochemistry for IL-11 and IL-11Rα
2.15.1 Cryostat sectioning
Biopsies were transported in liquid nitrogen to ensure that they did not thaw. The sections were cut using a Leica cryostat at -20°C. Each biopsy was removed from liquid nitrogen and attached to a holder using Cryo-M-Bed embedding compound. Serial cryostat sections of 10μm were obtained. Two sections were placed on each microscope slide and the duplicate section was used as a negative control in the immunocytochemistry. After cutting, the sections were
immediately fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at room temperature. The sections were then washed twice for 5 minutes in PBS and fixed in ice-cold methanol for 4 minutes then ice-cold acetone for 2 minutes. The sections were washed again for 2 x 5 minutes in PBS and stored in 8% sucrose solution (42.8g sucrose + 0.33g anhydrous MgCl₂ in 250ml 1x PBS + 250ml glycerol) at -20°C until required.

2.15.2 Principles of Immunocytochemistry

Immunocytochemistry uses antibodies to specifically bind to antigens present within the cell. An enzyme-labelled secondary antibody together with a substrate is then used to visualise where the antibodies have bound which produces a coloured product. In these studies, a VECTASTAIN ABC kit was used. The ABC method uses a biotinylated antibody and a preformed avidin biotinylated enzyme complex. In these experiments, a peroxidase enzyme was used. Because avidin has an extraordinarily high affinity for biotin, the binding of avidin to biotin is essentially irreversible. In addition, avidin has four binding sites for biotin, and most proteins, including enzymes, can be conjugated with several molecules of biotin which results in considerable amplification of the initial antibody-antigen binding. Sections of tissue were incubated with a primary antibody, which binds specifically to antigen that is present within the cell. The sections were then incubated with a biotinylated-secondary antibody in order to introduce many biotin molecules onto the sections at the location of the primary antibody. The avidin: biotinylated enzyme complex (ABC) (which will bind to the biotinylated secondary antibody) was then applied to the sections. The sections were then stained using a DAB kit (diamino-benzadine-tetrachloride is a peroxidase substrate which produces a brown coloured product in the presence of enzyme). This step allows the binding to be visualised. A counterstain is then used to aid visualisation of cells (Figure 2.2).
2.15.3 Immunocytochemistry protocol

Sections were washed twice for 5 minutes in PBS to remove sucrose solution and then incubated with blocking solution (100µl per section) 15µl blocking serum (either 15µl normal rabbit serum for IL-11Rα, or 15µl normal horse serum for IL-11 plus 5 drops avidin per ml PBS) for 30 minutes at room temperature. Sections were then incubated with 100µl of either IL-11Rα or IL-11 primary antibody overnight at 4°C. Each antibody was diluted (1:100) in PBS containing 15µl blocking serum and 5 drops biotin per ml. The sections were then washed twice in PBS for 5 minutes each, followed by incubation with 200µl biotinylated secondary antibody, diluted (1:200) in PBS containing 15µl blocking serum/ml, for 30 minutes at room temperature. Sections were washed twice again as before, and then incubated with 100µl avidin: biotinylated enzyme complex (Reagent A; Avidin DH, Reagent B; biotinylated horseradish peroxidase) which was diluted (1:50) in PBS for 30 minutes at room temperature. After repeating the wash twice, sections were stained for 8 minutes with DAB (Diamino-benzadine-tetrachloride) substrate to visualise antibody binding and counterstained for 10 minutes with haematoxylin (10% in water). Sections were then dehydrated through 50%, 70%, 90% and 95% alcohol for 5 minutes each and then absolute alcohol for 1-2 hours, followed by clearing in xylene overnight and mounted within DePeX. All incubations were carried out in a humid chamber. For each slide, one of the sections was used as a negative control where PBS was added instead of the primary antibody. Immunostaining for IL-11 and IL-11Rα were each repeated on at least 3 different sections from each biopsy.
Figure 2.2 Principles of immunocytochemistry using VECTASTAIN ABC kit (Taken from ABC catalogue).
2.16 Analysis of staining pattern and intensity by using H-SCORE

The intensity of staining for IL-11 and IL-11Rα in endometrial stromal and epithelial cells was assessed using the H-score method adapted by Lessey and others for analysis of immunocytochemistry staining in sections from endometrial biopsies (Lessey et al. 1996). The slides were scored independently by two observers. The final result for each stage of the menstrual cycle was obtained by taking the mean of each individual observer's results. Each individual observer's result was calculated from the analysis of ten fields at x 400 magnification. In the event of large differences between scores the section was reanalysed by both observers together. The H-score value was calculated according to the following equation:

\[ \text{H-SCORE} = \sum P_i (i+1) \]

Where \( i \) = staining intensity (1 = weak, 2 = moderate, 3 = strong) and \( P_i \) = percentage of cells staining at each intensity (0-100%).

2.17 Statistical analysis

Allele frequencies and carriage rates were compared and significance was assessed using the Pearson's \( \chi^2 \) test. Comparison of the means for IL-1β plasma levels in recurrent miscarriage women was carried out using student t-test in Microsoft EXCEL (paired, for equal variance). H-scores for IL-11 and IL-11Rα immunostaining and intensity ratios for IL-11 and IL-11Rα/GAPDH RT-PCR products was also carried out using student t-test in Microsoft EXECL. A P value < 0.05 was considered significant.

2.17.1 Hardy-Weinberg equilibrium

The Hardy-Weinberg equation was used to assess whether the observed genotype distribution differed significantly from that expected from a population in equilibrium.
The Hardy-Weinberg equation is: \( p^2 + q^2 + 2pq = 1 \)

Where \( p \) = frequency of common allele and \( q \) = frequency of rare allele. The equation gives the expected frequency of homozygotes \( (p^2 \) and \( q^2 \)) and heterozygotes \( (2pq) \). If the observed genotype distribution is not significantly different from the expected genotype distribution, then the genotype distribution is said to be consistent with that expected from a population in Hardy-Weinberg equilibrium. H-W analysis is used to give an indication of whether the sampling and genotyping methods are valid.

### 2.17.2 Pearson's chi-squared \((\chi^2)\) analysis

Chi-squared analysis tests the null hypothesis (that no difference between the observed and expected distributions). The value of \( \chi^2 \) is given by the equation:

\[
\chi^2 = \sum \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}
\]

The degrees of freedom equals the number of classes minus 1. For testing the Hardy-Weinberg expectations, comparison of genotypes and carriage rates between different groups (i.e. control Vs RM women) was carried out using contingency tables. For these, the degree of freedom is calculated by \((\text{number of rows minus 1}) \times (\text{number of columns minus 1})\). \( \chi^2 \) tables or CHIDIST in Microsoft EXCEL are then used to generate a p-value. A simple method to calculate \( \chi^2 \) can be employed using a 2 x 2 contingency table.
Table 2.1 An example of a 2 x 2 contingency table was used to calculate $\chi^2$

<table>
<thead>
<tr>
<th>Variable 2</th>
<th>Variable 1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carriage of allele 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-carriage of allele 4</td>
<td></td>
</tr>
<tr>
<td>RM group</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Control</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td>Total</td>
<td>a + c</td>
<td>b + d</td>
</tr>
</tbody>
</table>

$\chi^2$ can then be calculated from this equation:

$\chi^2 = (ad-bc)^2 / (a+b)(c+d)(b+d)(a+c)$

$\chi^2$ table are then used to determine the level of significances.
The null hypothesis was rejected and the statistic was considered significant if the p value was < 0.05.

2.17.3 Fisher's Exact Test For a 2 x 5 or smaller cross table

The website (http://home.clara.net/sisa/fiveby2.htm) was used to calculate significance of differences between groups for allele distribution using the Fisher Exact Test. The Fisher Exact Test was used because the numbers in some groups were less than five. The site also gives the Pearson's $\chi^2$ values. To compare carriage rates, 2 x 2 (dof = 1) contingency tables were used, to compare distributions of all 3 genotypes 3 x 2 (dof = 2) contingency tables were used. P-values for the different chi-squared contingency tables was also calculated using this website.

2.17.4 Carriage rate and allele frequency

Carriage rate and allele frequency were used to compare the difference in the distribution of alleles for the gene of interest in the patient group with the distribution of alleles in a control group. This shows whether any particular allele or genotype is over-represented or under-represented in patients. The data obtained for the recurrent miscarriage group of women were compared to that previously found in controls. The comparison of genotype frequencies between
controls and patients were used to determine whether any genotype (or carriage of a particular allele) is associated with susceptibility to recurrent miscarriage. Alleles associated with a disease might have a direct functional effect, or might be acting as markers of another, nearby, polymorphism in linkage disequilibrium.

2.17.5 F-test
The F-test was used to determine whether those was equal or unequal variance in values obtained in different groups. This enabled us to use the correct t-test.

2.17.6 Student's t-test
Using EXCEL software, the student's t-test was used to test the difference in means between two populations. Different types of t test are used, depending on the sample populations; a paired t test is used to test paired data, a t-test assuming equal variance is used when the sample data has an equal variance (for samples of equal or unequal variance was assessed by the F-test). This test was used when the data was normally distributed. The null hypothesis was rejected if the P (t) < 0.05 (statistically significant). The student's t test was used to determine if there were statistical differences between 1) plasma levels of IL-1β in recurrent miscarriage women with different genotypes, 2) H-scores for IL-11 and IL-11Rα staining in tissue obtained at different stages of the cycle in control women and in endometrial tissue obtained from control women and recurrent miscarriage women during the peri-implantation period, and 3) IL-11/GAPDH and IL-11Rα/GAPDH mRNA ratios in tissue obtained at different stages of the cycle from control women and in endometrial tissue from control women and recurrent miscarriage women during the peri-implantation period.
Chapter 3

The GLN223ARG Leptin Receptor Gene Polymorphism in Women with Recurrent Miscarriage

3.1 Introduction
3.1.1 Role of Leptin in Reproduction

A connection between obesity, reproduction and leptin is well established. Animal studies have shown that female ob/ob mice are not only obese but also sterile (Chehab et al., 1996; Cunningham et al., 1999). The ob/ob mutant female mouse does not produce an active form of leptin, and is acyclic and sterile. This sterility is reversed by treatment with recombinant leptin, but not by food restriction, suggesting that leptin itself is required for normal reproductive function. Several studies have suggested that this effect of leptin on reproduction is due to its interaction with the hypothalamic-pituitary-gonad axis and its ability to modulate LH and follicle stimulating hormone (FSH) production (Barash et al., 1997; Yu et al., 1997). In mice and/or humans the amount of body fat stored is known to influence fertility, indicating a link between adipose tissue and the reproductive system (Frisch, 1990). Leptin may be an important signal indicating the adequacy of nutritional status for reproductive function (Conway and Jacobs, 1997).

Leptin is known to interact with the hypothalamic-pituitary-ovarian function in humans. In vivo and in vitro studies have shown that leptin increases the plasma concentrations of luteinising hormone (LH) and follicle stimulating hormone (FSH) (Fink et al., 1998; Cunningham et al., 1999), and LH pulse frequency and amplitude (Licinio et al., 1998). In addition, levels of leptin have been shown to correlate with LH levels during the menstrual cycle (Teirmaa et al., 1998). As well as exerting effects on the hypothalamus, leptin may also act directly on reproductive organs. Leptin receptors have been found in the prostate and the ovaries. Leptin decreases steroid production by both ovarian granulosa and theca cells (Zachow et al., 1997). Recent studies in vivo and in vitro have shown that human endometrial epithelial cells also express the leptin receptor (Senaris et al., 1997; Quinton et al., 2003) and, therefore, leptin may also affect endometrial function directly.
3.1.2 Leptin and puberty
Leptin levels rise at the onset of puberty (Hassink et al., 1996) and fall in postmenopausal women. Leptin levels increase further during pubertal development in girls; in contrast levels decrease in boys and this decrease correlates with a rise in testosterone levels which suppress leptin production (Sinha and Caro, 1998). Studies from our laboratory has shown concentrations of leptin-binding activity (LBA) to be low at birth, to increase during the pre-pubertal years and then to fall during puberty and remain stable during adult life. Concentrations of LBA correlate well with Tamer stage of puberty indicating that LBA concentrations may also be important for reproductive function (Quinton et al., 1999b).

3.1.3 Leptin and menstrual cycle
In females, plasma leptin levels vary during the menstrual cycle. They rise during the transition from the follicular to the peri-ovulatory phase and peak in the luteal phase. Transition from the luteal to the luteal/ follicular phase results in a decrease in circulating leptin levels to values in the follicular phase (Quinton et al., 1999). There is some evidence to suggest that leptin levels are stimulated by steroids (Castracane et al., 1998). Leptin levels are increased in vitro fertilization treatment (IVF) cycles, where ovulation is stimulated by endogenous steroid treatment. However, other studies have shown that leptin concentrations are not influenced by oral contraceptives, indicating that oestrogen and/or progesterone do not influence peripheral serum leptin concentrations (Teirmaa et al., 1998).

3.1.4 Leptin and pregnancy
In humans, plasma levels of leptin increase during pregnancy and reach a peak at week 28, after which they remain at the same level until birth. This increase is independent of body mass index (BMI) values and is due to synthesis of leptin by the placenta (Senaris et al., 1997). A reduction in leptin levels is observed in women around the peri-partum period with a slight reduction in the 24 hours before delivery, and a highly significant reduction in the 24 hours after delivery (Matsuda et al., 1997; Tom et al., 1997). A progressive rise in serum leptin levels is then observed from 0 until 4 months in the postpartum period. These changes do not reflect any variation in BMI in the women. The observed rise in
leptin after delivery appears to be independent of foetal or placental influence and maternal lactation or hormonal changes. A progressive rise in leptin values was also observed at 1 and 2 years postpartum (Matsuda et al., 1997; Tom et al., 1997).

3.1.5 Leptin and recurrent miscarriage
Previous studies have suggested that low concentrations of maternal plasma leptin are associated with sub-optimal pregnancy outcomes (Mise et al., 1998), and fetal birth weight is correlated with neonatal cord blood leptin concentrations (Tamura et al., 1998). This has therefore prompted studies to investigate whether abnormal leptin levels are associated with recurrent miscarriage. Two studies have investigated concentrations of leptin in women with a history of recurrent miscarriage. In the first study leptin levels were shown to be decreased in recurrent miscarriage women compared to the normal women in the first trimester of pregnancy (Lage et al., 1999). However, this study may not reliable because leptin concentrations were measured subsequent to foetal loss and so low levels could be a consequence of miscarriage itself. In a more recent study from our laboratory, plasma leptin and leptin binding activity (LBA) were measured in 53 recurrent miscarriage women during early pregnancy, before miscarriage. The results from these studies showed that leptin concentrations were lower in women who subsequently miscarried compared with those who had a live birth. In contrast, there was no difference in LBA between both groups of women (Laird et al., 2001).

3.1.6 The GLN223ARG leptin receptor polymorphism
Single nucleotide polymorphisms (SNP) are the most common genetic variants. Considine et al. (1996) were the first to identify a single nucleotide polymorphism in the coding region of the leptin receptor gene, at codon 223 (GLN223ARG). This polymorphism results in the substitution of a glutamine with an arginine in the extracellular region of the leptin receptor.

The aim of this study was to investigate the distribution of the GLN223ARG alleles in women with recurrent miscarriage and compare this distribution with that already known for healthy women.
3.2 Material and methods

3.2.1 Human subjects
206 peripheral blood samples were obtained from recurrent miscarriage women who were attending the Jessop Hospital for Women in Sheffield. DNA was extracted by two methods described in chapter 2. The control blood samples (n = 225) had been collected previously from a community-based group of Caucasian postmenopausal women, from the Sheffield area, by Professor R. Eastell from Northern General Hospital, as part of an Osteoporosis epidemiology study. They had been previously genotyped by Dr Naomi Quinton. The reproductive status of these women was not recorded.

3.2.2 Genotyping GLN223ARG leptin receptor gene
The PCR to detect the GLN223ARG leptin receptor gene polymorphism in DNA extracted from the blood of 206 recurrent miscarriage women and the genotyping by restriction digest with MspI enzyme was carried out as described in chapter 2.
3.3 Results

Table 3.1 and 3.2 shows the concentrations and purity ratios for DNA extracted by the in-house method and the DNA isolation kit. Although the purity ratios were similar for each extraction method, the concentration of DNA obtained using the DNA isolation kit (Median = 487, range = 60-1560ng/μl) were higher than those obtained using the in-house method (Median = 232, range = 25-1120ng/μl). However, both methods produced sufficient DNA of a suitable quality for PCR analysis.

Figure 3.1 shows an agarose gel containing the genotyping results obtained for the GLN232ARG leptin receptor polymorphism. A single band of 416 bp shows the presence of allele A, while the presence of 2 bands of 251 and 165 bp shows the presence of allele G. There are 2 bands for the G allele because this product contains a digestion site for the Msp I enzyme, which is absent when A is present. Therefore, the PCR product containing the G allele is cleaved by Msp I and produces 2 products of lower molecular weight. Lanes 2, 9, 10, 11, in the top half of the gel and lanes 2, 3, 4, 5, 6, 8, 9, 10, 11, in the bottom half of the gel show samples from individuals heterozygous at this single nucleotide polymorphism. Lanes 3, 4, 5, 6, in the top half of the gel and lane 7 in the bottom half of the gel show samples from a person homozygous for allele A, and lane 7 in the top half of the gel shows a sample from a person homozygous for allele G.

Tables 3.3 and 3.4 shows the data for Hardy-Weinberg analysis and $\chi^2$ analyses of the distribution of genotypes for the GLN232ARG leptin receptor SNP in all 206 recurrent miscarriage women and in 225 control women. The balance of homozygotes and heterozygotes observed was as predicted by the Hardy-Weinberg equation from these allele frequencies. Table 3.5 and figure 3.2 shows the GLN232ARG leptin receptor genotyping and allele frequencies in controls and recurrent miscarriage women as a whole group. There was a slightly increased frequency of AA homozygotes and decreased frequency of heterozygotes in recurrent miscarriage women compared to control, but this was not significant.
Table 3.1 Concentrations of DNA and 260/280nm ratios obtained after DNA extracted from the first 102 blood samples using the in-house method.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>DNA conc.</th>
<th>260/280 nm ratio</th>
<th>Sample number</th>
<th>DNA conc.</th>
<th>260/280 nm ratio</th>
<th>Sample number</th>
<th>DNA conc.</th>
<th>260/280 nm ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200 ng/µl</td>
<td>2.1</td>
<td>47</td>
<td>135 ng/µl</td>
<td>1.8</td>
<td>93</td>
<td>350 ng/µl</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>215 ng/µl</td>
<td>1.9</td>
<td>48</td>
<td>185 ng/µl</td>
<td>1.2</td>
<td>94</td>
<td>275 ng/µl</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>150 ng/µl</td>
<td>2.1</td>
<td>49</td>
<td>110 ng/µl</td>
<td>1.4</td>
<td>95</td>
<td>215 ng/µl</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>90 ng/µl</td>
<td>2.7</td>
<td>50</td>
<td>360 ng/µl</td>
<td>1.2</td>
<td>96</td>
<td>330 ng/µl</td>
<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td>540 ng/µl</td>
<td>1.9</td>
<td>51</td>
<td>380 ng/µl</td>
<td>1.3</td>
<td>97</td>
<td>60 ng/µl</td>
<td>2.3</td>
</tr>
<tr>
<td>6</td>
<td>215 ng/µl</td>
<td>2.0</td>
<td>52</td>
<td>135 ng/µl</td>
<td>1.8</td>
<td>98</td>
<td>140 ng/µl</td>
<td>2.3</td>
</tr>
<tr>
<td>7</td>
<td>105 ng/µl</td>
<td>1.8</td>
<td>53</td>
<td>260 ng/µl</td>
<td>1.8</td>
<td>99</td>
<td>140 ng/µl</td>
<td>2.0</td>
</tr>
<tr>
<td>8</td>
<td>145 ng/µl</td>
<td>2.1</td>
<td>54</td>
<td>190 ng/µl</td>
<td>1.8</td>
<td>100</td>
<td>160 ng/µl</td>
<td>2.0</td>
</tr>
<tr>
<td>9</td>
<td>170 ng/µl</td>
<td>2.1</td>
<td>55</td>
<td>300 ng/µl</td>
<td>2.1</td>
<td>101</td>
<td>25 ng/µl</td>
<td>2.1</td>
</tr>
<tr>
<td>10</td>
<td>405 ng/µl</td>
<td>2.0</td>
<td>56</td>
<td>255 ng/µl</td>
<td>1.7</td>
<td>102</td>
<td>30 ng/µl</td>
<td>2.0</td>
</tr>
<tr>
<td>11</td>
<td>235 ng/µl</td>
<td>1.4</td>
<td>57</td>
<td>255 ng/µl</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>170 ng/µl</td>
<td>1.6</td>
<td>58</td>
<td>305 ng/µl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>100 ng/µl</td>
<td>2.4</td>
<td>59</td>
<td>290 ng/µl</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>310 ng/µl</td>
<td>1.9</td>
<td>60</td>
<td>900 ng/µl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>75 ng/µl</td>
<td>2.5</td>
<td>61</td>
<td>120 ng/µl</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>185 ng/µl</td>
<td>2.1</td>
<td>62</td>
<td>550 ng/µl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>115 ng/µl</td>
<td>1.8</td>
<td>63</td>
<td>130 ng/µl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>515 ng/µl</td>
<td>1.9</td>
<td>64</td>
<td>375 ng/µl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>110 ng/µl</td>
<td>2.2</td>
<td>65</td>
<td>195 ng/µl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>155 ng/µl</td>
<td>2.1</td>
<td>66</td>
<td>150 ng/µl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>925 ng/µl</td>
<td>1.8</td>
<td>67</td>
<td>110 ng/µl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>185 ng/µl</td>
<td>1.8</td>
<td>68</td>
<td>315 ng/µl</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>330 ng/µl</td>
<td>1.7</td>
<td>69</td>
<td>34 ng/µl</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>100 ng/µl</td>
<td>1.8</td>
<td>70</td>
<td>230 ng/µl</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>180 ng/µl</td>
<td>1.8</td>
<td>71</td>
<td>230 ng/µl</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>80 ng/µl</td>
<td>1.7</td>
<td>72</td>
<td>290 ng/µl</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>60 ng/µl</td>
<td>1.6</td>
<td>73</td>
<td>160 ng/µl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>255 ng/µl</td>
<td>1.8</td>
<td>74</td>
<td>305 ng/µl</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>230 ng/µl</td>
<td>1.7</td>
<td>75</td>
<td>30 ng/µl</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1120 ng/µl</td>
<td>1.8</td>
<td>76</td>
<td>100 ng/µl</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>50 ng/µl</td>
<td>2.1</td>
<td>77</td>
<td>140 ng/µl</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>215 ng/µl</td>
<td>1.7</td>
<td>78</td>
<td>175 ng/µl</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>305 ng/µl</td>
<td>1.7</td>
<td>79</td>
<td>285 ng/µl</td>
<td>2.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>110 ng/µl</td>
<td>1.6</td>
<td>80</td>
<td>70 ng/µl</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>165 ng/µl</td>
<td>2.0</td>
<td>81</td>
<td>205 ng/µl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>160 ng/µl</td>
<td>2.1</td>
<td>82</td>
<td>155 ng/µl</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>195 ng/µl</td>
<td>1.9</td>
<td>83</td>
<td>165 ng/µl</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>105 ng/µl</td>
<td>2.0</td>
<td>84</td>
<td>35 ng/µl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>25 ng/µl</td>
<td>2.0</td>
<td>85</td>
<td>345 ng/µl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>130 ng/µl</td>
<td>2.2</td>
<td>86</td>
<td>60 ng/µl</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>545 ng/µl</td>
<td>2.0</td>
<td>87</td>
<td>175 ng/µl</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>210 ng/µl</td>
<td>1.8</td>
<td>88</td>
<td>630 ng/µl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>135 ng/µl</td>
<td>1.7</td>
<td>89</td>
<td>380 ng/µl</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>265 ng/µl</td>
<td>1.7</td>
<td>90</td>
<td>580 ng/µl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>200 ng/µl</td>
<td>1.7</td>
<td>91</td>
<td>95 ng/µl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>230 ng/µl</td>
<td>1.5</td>
<td>92</td>
<td>295 ng/µl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2 Concentrations of DNA and 260/280nm ratios obtained from DNA extracted from the second set of blood samples using the DNA isolation kit (Gentra systems, USA).

<table>
<thead>
<tr>
<th>Sample number</th>
<th>DNA conc.</th>
<th>260/280 nm ratio</th>
<th>Sample number</th>
<th>DNA conc.</th>
<th>260/280 nm ratio</th>
<th>Sample number</th>
<th>DNA conc.</th>
<th>260/280 nm ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>103</td>
<td>195ng/μl</td>
<td>1.7</td>
<td>149</td>
<td>225ng/μl</td>
<td>1.5</td>
<td>195</td>
<td>205ng/μl</td>
<td>1.8</td>
</tr>
<tr>
<td>104</td>
<td>145ng/μl</td>
<td>1.9</td>
<td>150</td>
<td>100ng/μl</td>
<td>1.5</td>
<td>196</td>
<td>215ng/μl</td>
<td>1.9</td>
</tr>
<tr>
<td>105</td>
<td>95ng/μl</td>
<td>1.3</td>
<td>151</td>
<td>155ng/μl</td>
<td>1.3</td>
<td>197</td>
<td>205ng/μl</td>
<td>1.7</td>
</tr>
<tr>
<td>106</td>
<td>245ng/μl</td>
<td>2.0</td>
<td>152</td>
<td>525ng/μl</td>
<td>1.7</td>
<td>198</td>
<td>155ng/μl</td>
<td>2.0</td>
</tr>
<tr>
<td>107</td>
<td>95ng/μl</td>
<td>1.4</td>
<td>153</td>
<td>670ng/μl</td>
<td>1.6</td>
<td>199</td>
<td>570ng/μl</td>
<td>1.6</td>
</tr>
<tr>
<td>108</td>
<td>600ng/μl</td>
<td>1.9</td>
<td>154</td>
<td>340ng/μl</td>
<td>1.7</td>
<td>200</td>
<td>490ng/μl</td>
<td>1.7</td>
</tr>
<tr>
<td>109</td>
<td>360ng/μl</td>
<td>1.8</td>
<td>155</td>
<td>215ng/μl</td>
<td>1.4</td>
<td>201</td>
<td>665ng/μl</td>
<td>1.7</td>
</tr>
<tr>
<td>110</td>
<td>550ng/μl</td>
<td>2.0</td>
<td>156</td>
<td>390ng/μl</td>
<td>1.5</td>
<td>202</td>
<td>250ng/μl</td>
<td>1.6</td>
</tr>
<tr>
<td>111</td>
<td>735ng/μl</td>
<td>1.8</td>
<td>157</td>
<td>730ng/μl</td>
<td>1.7</td>
<td>203</td>
<td>190ng/μl</td>
<td>1.7</td>
</tr>
<tr>
<td>112</td>
<td>620ng/μl</td>
<td>1.7</td>
<td>158</td>
<td>665ng/μl</td>
<td>1.7</td>
<td>204</td>
<td>320ng/μl</td>
<td>1.6</td>
</tr>
<tr>
<td>113</td>
<td>225ng/μl</td>
<td>1.7</td>
<td>159</td>
<td>330ng/μl</td>
<td>1.3</td>
<td>205</td>
<td>195ng/μl</td>
<td>1.7</td>
</tr>
<tr>
<td>114</td>
<td>745ng/μl</td>
<td>1.7</td>
<td>160</td>
<td>380ng/μl</td>
<td>1.3</td>
<td>206</td>
<td>195ng/μl</td>
<td>1.8</td>
</tr>
<tr>
<td>115</td>
<td>1560ng/μl</td>
<td>1.7</td>
<td>161</td>
<td>1155ng/μl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>116</td>
<td>765ng/μl</td>
<td>1.7</td>
<td>162</td>
<td>975ng/μl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>117</td>
<td>420ng/μl</td>
<td>1.7</td>
<td>163</td>
<td>1020ng/μl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>118</td>
<td>1320ng/μl</td>
<td>1.7</td>
<td>164</td>
<td>555ng/μl</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>119</td>
<td>280ng/μl</td>
<td>1.7</td>
<td>165</td>
<td>1080ng/μl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>640ng/μl</td>
<td>1.7</td>
<td>166</td>
<td>885ng/μl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>110ng/μl</td>
<td>1.3</td>
<td>167</td>
<td>480ng/μl</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>122</td>
<td>470ng/μl</td>
<td>1.6</td>
<td>168</td>
<td>1240ng/μl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>123</td>
<td>835ng/μl</td>
<td>1.7</td>
<td>169</td>
<td>775ng/μl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>160ng/μl</td>
<td>1.9</td>
<td>170</td>
<td>80ng/μl</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>1055ng/μl</td>
<td>1.7</td>
<td>171</td>
<td>190ng/μl</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>126</td>
<td>660ng/μl</td>
<td>1.8</td>
<td>172</td>
<td>585ng/μl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>127</td>
<td>330ng/μl</td>
<td>1.8</td>
<td>173</td>
<td>325ng/μl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>455ng/μl</td>
<td>1.9</td>
<td>174</td>
<td>255ng/μl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>129</td>
<td>475ng/μl</td>
<td>1.7</td>
<td>175</td>
<td>230ng/μl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>220ng/μl</td>
<td>1.7</td>
<td>176</td>
<td>135ng/μl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>131</td>
<td>750ng/μl</td>
<td>1.8</td>
<td>177</td>
<td>895ng/μl</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>132</td>
<td>45ng/μl</td>
<td>1.5</td>
<td>178</td>
<td>390ng/μl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>133</td>
<td>1000ng/μl</td>
<td>1.8</td>
<td>179</td>
<td>545ng/μl</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>134</td>
<td>575ng/μl</td>
<td>1.5</td>
<td>180</td>
<td>315ng/μl</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>60ng/μl</td>
<td>1.7</td>
<td>181</td>
<td>335ng/μl</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>180ng/μl</td>
<td>1.6</td>
<td>182</td>
<td>1000ng/μl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>137</td>
<td>90ng/μl</td>
<td>1.4</td>
<td>183</td>
<td>715ng/μl</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>265ng/μl</td>
<td>1.6</td>
<td>184</td>
<td>690ng/μl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>139</td>
<td>420ng/μl</td>
<td>1.6</td>
<td>185</td>
<td>510ng/μl</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>195ng/μl</td>
<td>1.6</td>
<td>186</td>
<td>410ng/μl</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>141</td>
<td>125ng/μl</td>
<td>1.5</td>
<td>187</td>
<td>135ng/μl</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>142</td>
<td>570ng/μl</td>
<td>1.6</td>
<td>188</td>
<td>1280ng/μl</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>143</td>
<td>415ng/μl</td>
<td>1.7</td>
<td>189</td>
<td>285ng/μl</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>390ng/μl</td>
<td>1.6</td>
<td>190</td>
<td>650ng/μl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>145</td>
<td>410ng/μl</td>
<td>1.6</td>
<td>191</td>
<td>545ng/μl</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>146</td>
<td>445ng/μl</td>
<td>1.8</td>
<td>192</td>
<td>330ng/μl</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>147</td>
<td>555ng/μl</td>
<td>1.6</td>
<td>193</td>
<td>590ng/μl</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>148</td>
<td>365ng/μl</td>
<td>1.7</td>
<td>194</td>
<td>1425ng/μl</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1 An agarose gel showing the digested PCR products for GLN223ARG leptin receptor polymorphism genotyping.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Upper gel</th>
<th>Lower gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker (ΦX174 DNA/HaeIII)</td>
<td>Marker (ΦX174 DNA/HaeIII)</td>
</tr>
<tr>
<td>2</td>
<td>Heterozygote A G</td>
<td>Heterozygote A G</td>
</tr>
<tr>
<td>3</td>
<td>Homozygote AA</td>
<td>Heterozygote A G</td>
</tr>
<tr>
<td>4</td>
<td>Homozygote AA</td>
<td>Heterozygote A G</td>
</tr>
<tr>
<td>5</td>
<td>Homozygote AA</td>
<td>Heterozygote A G</td>
</tr>
<tr>
<td>6</td>
<td>Homozygote AA</td>
<td>Heterozygote A G</td>
</tr>
<tr>
<td>7</td>
<td>Homozygote GG</td>
<td>Homozygote AA</td>
</tr>
<tr>
<td>8</td>
<td>Homozygote AA</td>
<td>Heterozygote A G</td>
</tr>
<tr>
<td>9</td>
<td>Heterozygote A G</td>
<td>Heterozygote A G</td>
</tr>
<tr>
<td>10</td>
<td>Heterozygote A G</td>
<td>Heterozygote A G</td>
</tr>
<tr>
<td>11</td>
<td>Heterozygote A G</td>
<td>Heterozygote A G</td>
</tr>
<tr>
<td>12</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Table 3.3 Hardy-Weinberg analysis and Pearson's $\chi^2$ analysis of GLN223ARG polymorphism in 206 recurrent miscarriage women. $\chi^2 = 0.023$, at two degrees of freedom. $P = 0.99$

<table>
<thead>
<tr>
<th></th>
<th>Observed Genotype frequency</th>
<th>Expected Genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Heterozygotes AG</td>
<td>92</td>
<td>45</td>
</tr>
<tr>
<td>Homozygous AA</td>
<td>89</td>
<td>43</td>
</tr>
<tr>
<td>Homozygous GG</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>206</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

Table 3.4 Hardy-Weinberg analysis and Pearson's $\chi^2$ analysis of GLN223ARG polymorphism in control women. $\chi^2 = 0.95$, at two degrees of freedom. $P = 0.62$

<table>
<thead>
<tr>
<th></th>
<th>Observed Genotype frequency</th>
<th>Expected Genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Heterozygotes AG</td>
<td>115</td>
<td>48</td>
</tr>
<tr>
<td>Homozygous AA</td>
<td>78</td>
<td>36</td>
</tr>
<tr>
<td>Homozygous GG</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>225</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>
Table 3.5 Shows GLN223ARG leptin receptor genotype in control women and the whole group of recurrent miscarriage women. $\chi^2 = 3.31$, at two degrees of freedom. $P = 0.19$

<table>
<thead>
<tr>
<th>Genotype</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>Total</th>
<th>Allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>78</td>
<td>115</td>
<td>32</td>
<td>225</td>
<td>(A = 0.6, G = 0.4)</td>
</tr>
<tr>
<td>RM group</td>
<td>89</td>
<td>92</td>
<td>25</td>
<td>206</td>
<td>(A = 0.65, G = 0.35)</td>
</tr>
</tbody>
</table>
Figure 3.2 GLN223ARG leptin receptor genotypes in control women, and the whole group of recurrent miscarriage women.
Table 3.6 and figures 3.3 shows the distribution of GLN223ARG genotypes in recurrent miscarriage women with polycystic ovarian disease, thyroid abnormality, coagulation abnormality or uterine abnormality. The $\chi^2$ values were calculated in comparison with controls. $\chi^2$ analysis showed that there was no significant difference in the genotype distribution between controls and these four groups of recurrent miscarriage women.

Table 3.7 and figure 3.4 shows the distribution of GLN223ARG genotypes in women with unexplained recurrent miscarriage, recurrent miscarriage women with unknown cause and recurrent miscarriage women with retarded endometrium. $\chi^2$ analysis showed that there was a significantly decreased frequency of heterozygotes (AG) and an increased frequency of the (AA) homozygotes in recurrent miscarriage women in whom the cause of miscarriage is unknown, compared to the control women.

Table 3.8 and figure 3.5 shows the distribution of GLN223ARG genotypes in women divided according to whether they were suffering primary or secondary recurrent miscarriage. $\chi^2$ analysis showed that, there was a significantly increased frequency of the AA genotype and a significantly decreased frequency of the AG genotype in women with secondary recurrent miscarriage compared to control women. In contrast, there were no significant differences in genotype distribution between women with primary recurrent miscarriage compared to controls.
Table 3.6 Distribution of GLN223ARG genotypes in recurrent miscarriage women with polycystic ovarian disease, thyroid abnormality, coagulation abnormality or uterine abnormality compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>Total</th>
<th>$\chi^2$ dol = 2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCO</strong></td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>10</td>
<td>2.53</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>78</td>
<td>115</td>
<td>32</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>82</td>
<td>118</td>
<td>35</td>
<td>235</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Thyroid abnormality</strong></td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>16</td>
<td>1.75</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>78</td>
<td>115</td>
<td>32</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>84</td>
<td>121</td>
<td>36</td>
<td>241</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Coagulation abnormality</strong></td>
<td>15</td>
<td>20</td>
<td>6</td>
<td>41</td>
<td>0.08</td>
<td>0.97</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>78</td>
<td>115</td>
<td>32</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>93</td>
<td>135</td>
<td>38</td>
<td>266</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Uterine abnormality</strong></td>
<td>16</td>
<td>11</td>
<td>4</td>
<td>31</td>
<td>3.52</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>78</td>
<td>115</td>
<td>32</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>94</td>
<td>126</td>
<td>36</td>
<td>256</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.3 GLN223ARG genotypes in recurrent miscarriage women with polycystic ovarian disease, thyroid abnormality, coagulation abnormality or uterine abnormality.
### Table 3.7 Distribution of GLN223ARG genotypes in women with unexplained recurrent miscarriage, recurrent miscarriage women whose cause is unknown and recurrent miscarriage women with retarded endometrium, compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>Total</th>
<th>( \chi^2 )</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unexplained RM</strong></td>
<td>31</td>
<td>46</td>
<td>9</td>
<td>86</td>
<td>0.77</td>
<td>0.72</td>
</tr>
<tr>
<td>Control</td>
<td>78</td>
<td>115</td>
<td>32</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>109</td>
<td>161</td>
<td>41</td>
<td>311</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Unknown cause</strong></td>
<td>18</td>
<td>4</td>
<td>3</td>
<td>25</td>
<td>14.07</td>
<td>0.0006***</td>
</tr>
<tr>
<td>Control</td>
<td>78</td>
<td>115</td>
<td>32</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>96</td>
<td>119</td>
<td>35</td>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Retarded endometrium</strong></td>
<td>12</td>
<td>13</td>
<td>2</td>
<td>27</td>
<td>1.52</td>
<td>0.54</td>
</tr>
<tr>
<td>Control</td>
<td>78</td>
<td>115</td>
<td>32</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>90</td>
<td>128</td>
<td>34</td>
<td>252</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.4 GLN223ARG genotypes in recurrent miscarriage women in whom the cause of miscarriage is unexplained, recurrent miscarriage women whose cause is unknown and recurrent miscarriage women with retarded endometrium.
<table>
<thead>
<tr>
<th></th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>Total</th>
<th>$\chi^2$ &lt;br&gt; dof = 2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary miscarriage</td>
<td>42</td>
<td>57</td>
<td>11</td>
<td>110</td>
<td>1.29</td>
<td>0.54</td>
</tr>
<tr>
<td>Control</td>
<td>78</td>
<td>115</td>
<td>32</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>120</td>
<td>172</td>
<td>43</td>
<td>335</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary miscarriage</td>
<td>44</td>
<td>34</td>
<td>13</td>
<td>91</td>
<td>5.74</td>
<td>0.06*</td>
</tr>
<tr>
<td>Control</td>
<td>78</td>
<td>115</td>
<td>32</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>122</td>
<td>149</td>
<td>45</td>
<td>316</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.5 GLN223ARG genotypes in women with primary and secondary recurrent miscarriage.
Tables 3.9 and 3.10 show the carriage rates of allele A and allele G, and the $\chi^2$ analysis in recurrent miscarriage women as a whole group or when divided to various different sub-groups of miscarriage. The results show that the carriage rate of allele A was higher in the whole group of recurrent miscarriage women compared to controls, but it was not significant. $\chi^2$ analysis also showed significantly increased allele A frequencies in women with secondary recurrent miscarriage and in those in whom the cause of miscarriage is unknown compared to controls.

Together these results suggest a possible increased frequency of the A allele and the AA genotype in some women with recurrent miscarriage.
Table 3.9 The carriage rates of allele A and allele G in the recurrent miscarriage women as a whole group or when divided according to primary or secondary recurrent miscarriage.

<table>
<thead>
<tr>
<th></th>
<th>With G (GG + AG)</th>
<th>Without G (AA)</th>
<th>Total</th>
<th>$\chi^2$ d.o.f = 1</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM group</td>
<td>117</td>
<td>89</td>
<td>206</td>
<td>3.30</td>
<td>0.08</td>
</tr>
<tr>
<td>Control group</td>
<td>147</td>
<td>78</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>264</td>
<td>167</td>
<td>431</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary miscarriage</td>
<td>68</td>
<td>42</td>
<td>110</td>
<td>0.40</td>
<td>0.55</td>
</tr>
<tr>
<td>Control group</td>
<td>147</td>
<td>78</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>215</td>
<td>120</td>
<td>335</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary miscarriage</td>
<td>47</td>
<td>44</td>
<td>91</td>
<td>5.12</td>
<td>0.03*</td>
</tr>
<tr>
<td>Control</td>
<td>147</td>
<td>78</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>194</td>
<td>122</td>
<td>316</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.10 The carriage rates of allele A and allele G in the recurrent miscarriage women when divided to various different sub-group of miscarriage.

<table>
<thead>
<tr>
<th></th>
<th>With G (GG + AG)</th>
<th>Without G (AA)</th>
<th>Total</th>
<th>$\chi^2$ d.o.f = 1</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine abnormality</td>
<td>15</td>
<td>16</td>
<td>31</td>
<td>3.37</td>
<td>0.08</td>
</tr>
<tr>
<td>Control group</td>
<td>147</td>
<td>78</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>162</td>
<td>94</td>
<td>256</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown cause</td>
<td>7</td>
<td>18</td>
<td>25</td>
<td>13.26</td>
<td>0.0004***</td>
</tr>
<tr>
<td>Control group</td>
<td>147</td>
<td>78</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>154</td>
<td>96</td>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retarded endometrium</td>
<td>15</td>
<td>12</td>
<td>27</td>
<td>1.004</td>
<td>0.40</td>
</tr>
<tr>
<td>Control</td>
<td>147</td>
<td>78</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>162</td>
<td>90</td>
<td>252</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unexplained</td>
<td>55</td>
<td>31</td>
<td>86</td>
<td>0.05</td>
<td>0.89</td>
</tr>
<tr>
<td>Control group</td>
<td>147</td>
<td>78</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>202</td>
<td>109</td>
<td>311</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid abnormality</td>
<td>10</td>
<td>6</td>
<td>16</td>
<td>0.053</td>
<td>0.79</td>
</tr>
<tr>
<td>Control</td>
<td>147</td>
<td>78</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>157</td>
<td>84</td>
<td>241</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCO</td>
<td>6</td>
<td>4</td>
<td>10</td>
<td>0.12</td>
<td>0.74</td>
</tr>
<tr>
<td>Control group</td>
<td>147</td>
<td>78</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>153</td>
<td>82</td>
<td>235</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulation</td>
<td>26</td>
<td>15</td>
<td>41</td>
<td>0.06</td>
<td>0.86</td>
</tr>
<tr>
<td>Control</td>
<td>147</td>
<td>78</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>173</td>
<td>93</td>
<td>266</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4 Discussion

In this study, we investigated the distribution of alleles of the leptin receptor GLN223ARG polymorphism in women with recurrent miscarriage and compared this with that already known for a control Caucasian population. The $\chi^2$ results demonstrated that although the AA genotype was more common in women with recurrent miscarriage than in the general population, there was no significant difference between genotype distribution in controls and women with recurrent miscarriage as a whole group.

However, when the women were divided according to the cause of miscarriage, there was a significant increase in AA genotype in women with secondary recurrent miscarriage and in women whose cause of repeated miscarriage is unknown. The increase in AA genotype in women with secondary miscarriage and women with unknown cause was associated with a decrease in AG genotype compared to controls. This difference in genotype distribution in controls and some groups of women with recurrent miscarriage suggests that leptin signalling may play a role in the prevention of recurrent miscarriage. This polymorphism in the leptin receptor gene creates an amino acid change from arginine to glutamine in the extracellular domain of the receptor. This may result in altered leptin binding and therefore, receptor dimerisation and signalling capacity of the leptin receptor. However, a reason for differences seen only in women with secondary miscarriage and in women in whom the cause for miscarriage is unknown is difficult to explain.

Several studies have investigated the distribution of the GLN223ARG polymorphism in various groups, but found no association with obesity (Matsuoka et al., 1997) body mass index and plasma insulin (Gotoda et al., 1997). However an association between homozygosity of the G allele for this polymorphism and low leptin levels in a cohort of Pima Indians has been reported (Thompson et al., 1997, abstract). Recent studies (Quinton et al., 1998) have reported an association between homozygosity of the G allele of the leptin receptor GLN223ARG and leptin levels, fat mass and body mass index (BMI) in a population of normal postmenopausal Caucasian women.
Chagnon et al. (2000) have also reported similar findings of associations between body mass index and fat mass and the polymorphism GLN223ARG in a population of Caucasian middle-aged men. In their study, carriers of the A allele, had higher BMI, fat mass, percent fat mass and leptin than non-carriers. They suggest that the leptin receptor gene variation has a significant effect on adiposity in Caucasian male subjects aged 44-46 years.

Previous studies reported that abnormally low serum leptin levels are observed in women after suffering recurrent miscarriage in the first trimester of pregnancy (Lage et al., 1999) and that there is increasing evidence for a role of leptin in reproduction and particularly successful pregnancy outcome (Quinton et al., 1999; Ashworth et al., 2000; Barash et al., 1996; Butte et al., 1997). A recent study has reported lower leptin concentrations in recurrent miscarriage women who subsequently miscarried compare with those who had a live birth. In contrast, there was no difference in leptin binding activity between both groups of women (Laird et al., 2001). Leptin appears to be an important regulator of foetal growth as many studies have shown that low concentrations of maternal plasma leptin are associated with sub-optimal pregnancy outcomes (Lage et al., 1999; Lea et al., 2000; Laird et al., 2001).

The mechanism by which leptin may affect pregnancy outcome is unknown. Leptin is produced by the placenta, and there are a number of possible effects that it may have on placental growth. Leptin is known to interact with cytokine networks (Granowitz, 1997; Yamaguchi et al., 1998) and, therefore, leptin may affect pregnancy outcome by affecting the balance of cytokines in the foetal-placental unit. More recent studies have shown leptin receptor expression in endothelial cells of various foetal blood vessels of chorionic villi during the first trimester of gestation (Castellucci et al., 2000). Leptin may therefore, contribute to the development of placental vessels, together with other growth factors such as vascular endothelial growth factor (Shiraishi et al., 1996), basic fibroblast growth factor (Mühlhauser et al., 1996) and platelet derived growth factor B (Holmgren et al., 1991).
Recent studies have also shown that the long isoform of the leptin receptor is expressed in the human endometrium throughout the menstrual cycle (Alfer et al., 2000). In addition, Quinton et al. (2003) have reported the expression of the long isoform of the leptin receptor by cultured endometrial epithelial cells, and both cultured endometrial stromal and epithelial cells expressed the short and variant isoforms of the receptor. Some studies have also shown that leptin is produced by the endometrium (Gonzalez et al., 2001) although other studies have suggested that it is not (Alfer et al., 2000).

The results of this thesis are suggestive of a role for leptin in feto-placental development and recurrent miscarriage, but because significant differences in allele distribution were only seen in women with secondary recurrent miscarriage and in women in whom the cause is unknown, a much larger study is needed in order to determine whether the association observed in this project are reproducible.
Chapter 4

Interleukin-1 Receptor Antagonist and Interleukin-1 Beta Polymorphisms in Women with Recurrent Miscarriage

4.1 Introduction
4.1.1 The interleukin-1 system

The human interleukin-1 family of proteins comprise the products of nine genes, which are clustered together on chromosome 2 (Nicklin et al., 2002). The classical family members, IL-1α (159 amino acids) and IL-1β (153 amino acids) are two structurally related polypeptides, encoded by separate genes (respectively, IL1A and IL1B). IL-1 receptor antagonist (IL-1ra) (152 amino acids) which is encoded by the gene IL1RN is an anti-inflammatory nonsignaling molecule that competes for receptor binding with IL-1α and IL-1β. There are two types of IL-1 receptor, IL-1 receptor type I which is the functional receptor and type II which is non-functional.

4.1.2 IL-1 and endometrial function

Numerous studies have shown the expression of the various components of the IL-1 system in the endometrium. In both mouse and human endometrium, IL-1β and IL-1α have been localized at the mRNA and protein levels to endometrial macrophages and endothelial cells (Tackac et al. 1988). Other studies (Simon et al. 1993) have also shown its presence in epithelial and stromal cells in human endometrium.

The IL-1 receptor type I (IL-1Rtl) mRNA is expressed in the human endometrium throughout the menstrual cycle, reaching maximal levels in the early and late luteal phases. Expression is higher in human endometrial epithelial cells than in stromal cells. IL-1α and β and IL-1ra are also expressed by the blastocyst. The shared presence of the IL-1 system in maternal and embryonic tissues during early human implantation supports an autocrine/paracrine role for the IL-1 system in human implantation (Simon et al., 1994a, 1997).
IL-1 has been shown to affect the production of numerous factors thought to be involved in embryo implantation and placental growth including MMP's (Meisser et al., 1999 a, b), LIF (Laird et al., 1997) and integrins (Sillem, et al., 1999). Successful implantation after in vitro fertilization has also been correlated with high concentrations of both IL-1α and IL-1β in the culture medium of human embryos (Sheth et al., 1991; Baranao et al., 1997; Karagouni et al., 1998).

4.1.3 Interleukin-1 receptor antagonist and endometrial function

There are two forms of IL-1ra, a secreted and an intracellular one. The secreted form of IL-1ra is present in the human endometrium. Studies have shown that IL-1ra is present in the endometrium throughout the entire menstrual cycle and is located primarily in the endometrial epithelium (Simon et al., 1993). In addition, stromal and glandular cells express the intracellular form of IL-1ra (icIL-1ra).

4.1.4 Interleukin-1 receptor antagonist gene polymorphism

The variable number tandem repeat (VNTR) polymorphism in intron 2 of the IL1RN gene results in the presence of 2-6 repeats of an 86bp sequence. The frequency of these repeats in the general population is shown in (Table 4.1). Despite the fact that these differences are present in intron 2 of the IL1RN gene the presence of different repeats have also been associated with altered production of IL-1β in mononuclear cells (Santtila et al. 1998).

4.1.5 Interleukin-1β-511 gene polymorphism

The polymorphism at position -511 is a substitution of cytosine by thymine in the promoter region of the IL1B gene. 43.5% of individuals in a northern England population are heterozygous at this position (Read et al. 2000).

The aim of this study was to type the IL-1B-511 C/T and the IL-1RN tandem repeat polymorphism in 206 recurrent miscarriage women with various known causes of recurrent miscarriage and compare their distribution with that already known for an ethnically-matched control population.
4.2 Material and methods

4.2.1 Human subjects

This study used the same blood obtained from 206 women attending the recurrent miscarriage clinic at the Jessop Hospital for Women in Sheffield as was used in the leptin receptor polymorphism study. The entry criteria were described in material and methods in chapter 2. The blood samples were collected with anticoagulant and DNA was extracted by the two methods described in chapter 2. The control blood samples used for IL1RN gene polymorphism (n = 259), and for IL1B gene polymorphism (n = 224) were collected from unrelated Caucasian men and women from the Sheffield and Manchester areas (blood and sperm donors).

4.2.2 PCR-analysis for IL1RN and IL1B polymorphisms

The PCR reactions to detect the IL1RN and IL1B polymorphisms are described in chapter 2. The restriction enzyme Ava I was used to detect genotype at the IL1B polymorphism, by digestion of the PCR product. The genotype and allele distribution frequencies for IL1RN gene and IL1B-511 polymorphisms were compared with those already known for the control population (A.I.F. Blakemore, personal communication).

4.2.3 Plasma IL-1β concentration

The amount of IL-1β in the plasma of blood samples taken from the same recurrent miscarriage women were measured using a QuantiKine immunoassay kit (R&D Systems, Abingdon, UK.), as described in chapter 2. The IL-1β levels in the plasma were compared between patients with different genotypes for the IL1RN and IL1B genes.

4.2.4 Electrophoresis

Agarose gel electrophoresis was carried out as described in chapter 2 to show the different PCR products for the IL1RN polymorphism. Polyacrylamide gel electrophoresis was carried out as described in chapter 2 to separate the PCR products for IL1B polymorphism in the same DNA samples. The products of the IL1B PCR are better resolved by polyacrylamide gel in electrophoresis which gives clearer and stronger bands.
4.3 Results

4.3.1 IL1RN gene polymorphism

In our study, only 2 women were heterozygous for 5-repeat alleles and no 3-repeat alleles were found. Thus, the analysis is limited to women with either 2 or 4 copies of the 86 bp sequence. Figure 4.1 shows a gel demonstrating results obtained from recurrent miscarriage women with 2,2 - 4,4 and 4,2 genotypes of the IL1RN gene. A sample from a woman with a 2, 2 genotype produces a single band of molecular weight 240 bp (e.g. lane 1 in the gel), a sample from a woman with 4, 4 genotype shows a single band of 410 bp (e.g. lane 9 in the gel) and a sample from a woman with a 4, 2 genotype produces two bands of 410 bp and 240 bp (e.g. lane 2 in the gel). No bands were seen in lane 8 in the gel showing that the PCR had not worked. However, bands were seen in later gels when the PCR reaction was repeated with increased amounts of DNA.

Table 4.1 and 4.2 shows the data for Hardy-Weinberg and $\chi^2$ analysis of genotype distributions for IL1RN in the whole population of 206 recurrent miscarriage women and in controls. The balance of homozygotes and heterozygotes observed was as predicted by the Hardy-Weinberg equation from these allele frequencies.

Table 4.3 and figure 4.2 shows the distribution of genotypes for IL1RN and allele 4 and 2 frequencies in controls and the whole group of recurrent miscarriage women. $\chi^2$ analysis indicated that there was no significant difference between the genotype distribution in controls and in the whole group of recurrent miscarriage women.
Figure 4.1 An agarose gel showing the PCR products for IL1RN gene polymorphism.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker (ΦX174 DNA/HaeIII)</td>
</tr>
<tr>
<td>2</td>
<td>Homozygous band 2 2</td>
</tr>
<tr>
<td>3</td>
<td>Heterozygous bands 4 2</td>
</tr>
<tr>
<td>4</td>
<td>Heterozygous bands 4 2</td>
</tr>
<tr>
<td>5</td>
<td>Heterozygous bands 4 2</td>
</tr>
<tr>
<td>6</td>
<td>Heterozygous bands 4 2</td>
</tr>
<tr>
<td>7</td>
<td>Heterozygous bands 4 2</td>
</tr>
<tr>
<td>8</td>
<td>No bands</td>
</tr>
<tr>
<td>9</td>
<td>Homozygous band 4 4</td>
</tr>
<tr>
<td>10</td>
<td>Heterozygous bands 4 2</td>
</tr>
<tr>
<td>11</td>
<td>Heterozygous bands 4 2</td>
</tr>
<tr>
<td>12</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Allele 4 (410bp)
Allele 2 (240bp)
Table 4.1 Hardy-Weinberg equation and Pearson's $\chi^2$ analysis of IL1RN polymorphism in 206 recurrent miscarriage women. $\chi^2 = 0.013$, at two degree of freedom.  $P = 0.99$

<table>
<thead>
<tr>
<th>Observed Genotype frequency</th>
<th>Expected Genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Heterozygous 42</td>
<td>79</td>
</tr>
<tr>
<td>Homozygous 44</td>
<td>115</td>
</tr>
<tr>
<td>Homozygous 22</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>206</td>
</tr>
</tbody>
</table>

Table 4.2 Hardy-Weinberg equation and Pearson's $\chi^2$ analysis of IL1RN polymorphism in controls. $\chi^2 = 0.392$, at two degree of freedom.  $P = 0.82$

<table>
<thead>
<tr>
<th>Observed genotype frequency</th>
<th>Expected genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Heterozygous 42</td>
<td>92</td>
</tr>
<tr>
<td>Homozygous 44</td>
<td>150</td>
</tr>
<tr>
<td>Homozygous 22</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>259</td>
</tr>
</tbody>
</table>
Table 4.3 IL1RN genotyping in controls and the whole group of recurrent miscarriage women. $\chi^2 = 0.44$, at two degree of freedom. $P = 0.82$

<table>
<thead>
<tr>
<th>Genotype</th>
<th>22</th>
<th>42</th>
<th>44</th>
<th>Total</th>
<th>Allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM group</td>
<td>12</td>
<td>79</td>
<td>115</td>
<td>206</td>
<td>(2 = 0.25, 4 = 0.75)</td>
</tr>
<tr>
<td>Control group</td>
<td>17</td>
<td>92</td>
<td>150</td>
<td>259</td>
<td>(2 = 0.24, 4 = 0.76)</td>
</tr>
</tbody>
</table>
Figure 4.2 IL1RN genotypes in controls and the whole group of recurrent miscarriage women
Tables 4.4 and 4.5 show the distribution of genotypes for IL1RN in 206 recurrent miscarriage women, divided according to the cause of miscarriage. This is also illustrated graphically in figures 4.3 and 4.4. $\chi^2$ analyses showed that there was no significant difference between the genotype distribution in controls and in all groups of recurrent miscarriage women when divided into those with any particular cause of miscarriage except for women with PCOS. In recurrent miscarriage women with PCOS there was a significant increase in the frequency of the 2,2 and 4,2 genotypes and a decrease in frequency of the 4,4 genotype compared to control. However, the number of women in this group was small (10) and therefore this result should be treated with caution.

Table 4.6 and figure 4.5 show the distribution of genotypes for IL1RN gene in women with primary and secondary recurrent miscarriage. Again, no significant differences in genotype distribution were seen compared to the control population.
Table 4.4 The distribution of genotypes for the IL1RN polymorphism in recurrent miscarriage women with polycystic ovarian disease, thyroid abnormality, coagulation abnormality or uterine abnormality compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>44</th>
<th>42</th>
<th>22</th>
<th>Total</th>
<th>$\chi^2$ (dof = 2)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCOS</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>10</td>
<td>6.49</td>
<td>0.033**</td>
</tr>
<tr>
<td>Control</td>
<td>150</td>
<td>92</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>152</td>
<td>98</td>
<td>19</td>
<td>269</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid abnormality</td>
<td>10</td>
<td>4</td>
<td>2</td>
<td>16</td>
<td>1.30</td>
<td>0.45</td>
</tr>
<tr>
<td>Control</td>
<td>150</td>
<td>92</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>160</td>
<td>96</td>
<td>19</td>
<td>275</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulation abnormality</td>
<td>21</td>
<td>17</td>
<td>3</td>
<td>41</td>
<td>0.65</td>
<td>0.71</td>
</tr>
<tr>
<td>Control</td>
<td>150</td>
<td>92</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>171</td>
<td>109</td>
<td>20</td>
<td>290</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterine abnormality</td>
<td>21</td>
<td>9</td>
<td>1</td>
<td>31</td>
<td>1.29</td>
<td>0.67</td>
</tr>
<tr>
<td>Control</td>
<td>150</td>
<td>92</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>171</td>
<td>101</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.3 IL1RN genotypes in recurrent miscarriage women with polycystic ovarian disease, thyroid abnormality, coagulation abnormality or uterine abnormality.
Table 4.5 The distribution of genotypes for IL1RN polymorphism in women with unexplained recurrent miscarriage, recurrent miscarriage women whose cause is unknown and recurrent miscarriage women with retarded endometrium compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>44</th>
<th>42</th>
<th>22</th>
<th>Total</th>
<th>$\chi^2$ (dof = 2)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unexplained RM</td>
<td>47</td>
<td>35</td>
<td>3</td>
<td>85</td>
<td>1.7</td>
<td>0.48</td>
</tr>
<tr>
<td>Control</td>
<td>150</td>
<td>92</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>197</td>
<td>127</td>
<td>20</td>
<td>344</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown cause</td>
<td>16</td>
<td>8</td>
<td>2</td>
<td>26</td>
<td>0.25</td>
<td>0.85</td>
</tr>
<tr>
<td>Control</td>
<td>150</td>
<td>92</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>166</td>
<td>100</td>
<td>19</td>
<td>285</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retarded endometrium</td>
<td>15</td>
<td>11</td>
<td>1</td>
<td>27</td>
<td>0.53</td>
<td>0.80</td>
</tr>
<tr>
<td>Control</td>
<td>150</td>
<td>92</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>165</td>
<td>103</td>
<td>18</td>
<td>266</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.4 IL1RN genotypes in recurrent miscarriage women in whom the cause of miscarriage is unexplained or unknown and recurrent miscarriage women with retarded endometrium.
Table 4.6 The distribution of genotypes for IL1RN polymorphism in recurrent miscarriage women with primary and secondary recurrent miscarriage compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>44</th>
<th>42</th>
<th>22</th>
<th>Total</th>
<th>$\chi^2$ (dof = 2)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary miscarriage</td>
<td>63</td>
<td>42</td>
<td>5</td>
<td>110</td>
<td>0.68</td>
<td>0.76</td>
</tr>
<tr>
<td>Control</td>
<td>150</td>
<td>92</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>213</td>
<td>134</td>
<td>22</td>
<td>369</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                  | 50 | 35 |  6 |  91   | 0.26              | 0.87    |
| Secondary miscarriage | 150|  92| 17 |  259  |                   |         |
| Total            | 200| 127| 23 |  350  |                   |         |
Figure 4.5 IL1RN genotype in women with primary or secondary recurrent miscarriage.
Table 4.7 and 4.8 shows the carriage rate of allele 2 and allele 4, and the $\chi^2$ analysis results in the recurrent miscarriage women as a whole group or when divided to various different sub-groups depending on the cause of miscarriage in comparison with the control local population. The results show that there were also no significant differences for the carriage rates for either the 2 or 4 allele in all of the sub-groups of miscarriage patients compared to control local population.

Taken together, these results suggest that there was no difference between allele frequencies for the IL1RN polymorphisms in this population of recurrent miscarriage women and the local control population.
Table 4.7 The carriage rate of allele 2 and allele 4 in the recurrent miscarriage women as a whole group or when divided according to whether they suffer primary or secondary recurrent miscarriage.

<table>
<thead>
<tr>
<th></th>
<th>With 4</th>
<th>Without 4</th>
<th>Total</th>
<th>χ² (dof = 1)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM group</td>
<td>194</td>
<td>12</td>
<td>206</td>
<td>0.11</td>
<td>0.85</td>
</tr>
<tr>
<td>Control</td>
<td>242</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>436</td>
<td>29</td>
<td>465</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary miscarriage</td>
<td>105</td>
<td>5</td>
<td>110</td>
<td>0.56</td>
<td>0.63</td>
</tr>
<tr>
<td>Control</td>
<td>242</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>347</td>
<td>22</td>
<td>369</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary miscarriage</td>
<td>85</td>
<td>6</td>
<td>91</td>
<td>0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>Control</td>
<td>242</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>327</td>
<td>23</td>
<td>350</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.8 The carriage rate of allele 2 and allele 4 in the recurrent miscarriage women when divided to various different sub-groups according to the cause of miscarriage.

<table>
<thead>
<tr>
<th></th>
<th>With 4 (44+ 42)</th>
<th>Without 4 (22)</th>
<th>Total</th>
<th>(\chi^2) (dof = 1)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine abnormality</td>
<td>30</td>
<td>1</td>
<td>31</td>
<td>0.53</td>
<td>0.70</td>
</tr>
<tr>
<td>Control</td>
<td>242</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>272</td>
<td>18</td>
<td>290</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unexplained</td>
<td>82</td>
<td>3</td>
<td>85</td>
<td>1.08</td>
<td>0.43</td>
</tr>
<tr>
<td>Control</td>
<td>242</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>324</td>
<td>20</td>
<td>344</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retarded endometrium</td>
<td>26</td>
<td>1</td>
<td>27</td>
<td>0.34</td>
<td>1.00</td>
</tr>
<tr>
<td>Control</td>
<td>242</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>268</td>
<td>18</td>
<td>286</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown cause</td>
<td>24</td>
<td>2</td>
<td>26</td>
<td>0.05</td>
<td>0.69</td>
</tr>
<tr>
<td>Control</td>
<td>242</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>266</td>
<td>19</td>
<td>285</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid abnormality</td>
<td>14</td>
<td>2</td>
<td>16</td>
<td>0.83</td>
<td>0.31</td>
</tr>
<tr>
<td>Control</td>
<td>242</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>256</td>
<td>19</td>
<td>275</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCO</td>
<td>8</td>
<td>2</td>
<td>10</td>
<td>2.65</td>
<td>0.15</td>
</tr>
<tr>
<td>Control</td>
<td>242</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>19</td>
<td>269</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulation</td>
<td>38</td>
<td>3</td>
<td>41</td>
<td>0.03</td>
<td>0.74</td>
</tr>
<tr>
<td>Control</td>
<td>242</td>
<td>17</td>
<td>259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>280</td>
<td>20</td>
<td>300</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.2 IL1B gene polymorphisms

Figure 4.6 shows a gel showing the products obtained for the IL1B polymorphism. A single band of 304 bp showed the presence of the T allele, while the presence of 2 bands of 190 bp and 114 bp showed the presence of the C allele. There are 2 bands for the C allele because this product contains a digestion site for the Ava I enzyme, which is absent in the T allele. Therefore, the PCR product containing the C allele is cleaved by Ava I enzyme and produces 2 products of lower molecular weight. Lanes 2, 3, 7 and 8 show a heterozygote (T, C), lane 4 shows a homozygote for T and lanes 5, 6 and 9 show homozygotes for C. Lane 10 shows no PCR product, but when repeated with increased amounts of DNA bands were seen.

Tables 4.9 and 4.10 show the data for Hardy-Weinberg and $\chi^2$ analyses for the genotype distribution for IL1B in the total group of recurrent miscarriage women and controls. In the recurrent miscarriage group of women, the IL1B genotype distributions were not as predicted for the allele frequencies in a population in Hardy-Weinberg equilibrium, and this was due to an increased frequency of the heterozygous genotype. One reason for this might have been incomplete digestion of the PCR products, so that CC genotypes were showing a CT genotypes. To check this, the PCR analysis was repeated several times, and the same results was obtained on all occasions.

Table 4.11 and figure 4.7 show the distribution of genotypes for IL1B and allele C and T frequencies in controls and recurrent miscarriage women as a whole group. Although the heterozygote genotype (CT) was increased in the recurrent miscarriage group compared to controls, $\chi^2$ analysis showed that there was no significant difference between the genotype distribution in controls and recurrent miscarriage women. The allele frequencies were also very similar in both groups of women.
Figure 4.6 A polyacrylamide gel showing the Ava-I digested PCR products for IL1B gene polymorphism.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker (ΦX174 DNA/HaeIII)</td>
</tr>
<tr>
<td>2</td>
<td>Heterozygous bands CT</td>
</tr>
<tr>
<td>3</td>
<td>Heterozygous bands CT</td>
</tr>
<tr>
<td>4</td>
<td>Homozygous band TT</td>
</tr>
<tr>
<td>5</td>
<td>Homozygous band CC</td>
</tr>
<tr>
<td>6</td>
<td>Homozygous band CC</td>
</tr>
<tr>
<td>7</td>
<td>Heterozygous bands CT</td>
</tr>
<tr>
<td>8</td>
<td>Heterozygous bands CT</td>
</tr>
<tr>
<td>9</td>
<td>Homozygous band CC</td>
</tr>
<tr>
<td>10</td>
<td>Negative</td>
</tr>
</tbody>
</table>

(T) 304 bp  
(C) 190 bp  
(C) 114 bp
Table 4.9 Hardy-Weinberg analysis and Pearson’s $\chi^2$ analysis for IL1B polymorphism in 206 recurrent miscarriage women. $\chi^2 = 9.00$, at two degree of freedom. $P = 0.01$

<table>
<thead>
<tr>
<th>Genotype frequency</th>
<th>Expected Genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>117</td>
</tr>
<tr>
<td>Homozygous C</td>
<td>69</td>
</tr>
<tr>
<td>Homozygous T</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>206</td>
</tr>
</tbody>
</table>

Table 4.10 Hardy-Weinberg analysis and Pearson’s $\chi^2$ analysis for IL1B polymorphism in controls. $\chi^2 = 0.57$, at two degree of freedom. $P = 0.75$

<table>
<thead>
<tr>
<th>Genotype frequency</th>
<th>Expected genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Heterozygous CT</td>
<td>110</td>
</tr>
<tr>
<td>Homozygous CC</td>
<td>85</td>
</tr>
<tr>
<td>Homozygous TT</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>224</td>
</tr>
</tbody>
</table>
Table 4.11 The distribution of genotypes for the IL1B gene in controls and the whole group of recurrent miscarriage women. $\chi^2 = 2.78$, at two degree of freedom. $P = 0.26$

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TT</th>
<th>CT</th>
<th>CC</th>
<th>Total</th>
<th>Allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM group</td>
<td>20</td>
<td>117</td>
<td>69</td>
<td>206</td>
<td>(C = 0.62, T = 0.38)</td>
</tr>
<tr>
<td>Control group</td>
<td>29</td>
<td>110</td>
<td>85</td>
<td>224</td>
<td>(C = 0.62, T = 0.38)</td>
</tr>
</tbody>
</table>
Figure 4.7 IL1B genotypes in controls and in the whole group of recurrent miscarriage women.
Table 4.12 and 4.13 show the distribution of genotypes for IL1B in 206 recurrent miscarriage women, divided according to the causes of miscarriage. This is also demonstrated graphically in figure 4.8 and 4.9. $\chi^2$ analysis showed that there was no significant difference between the genotype distribution in controls and recurrent miscarriage women when divided according to the cause of miscarriage.

Table 4.14 and figure 4.10 show the distribution of genotypes for the IL1B gene in women with primary and secondary miscarriage. For the primary recurrent miscarriage women, the frequency of heterozygosity was increased and this gave rise to an increased $\chi^2$ value (4.93 at two degree of freedom, $P = 0.09$), but this was still not significant. This higher frequency of heterozygote genotype agrees with the increased frequency seen in the Hardy-Weinberg analysis.
Table 4.12 The distribution of genotypes for the IL1B polymorphism in recurrent miscarriage women with polycystic ovarian disease, thyroid abnormality, coagulation abnormality or uterine abnormality compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Total</th>
<th>$\chi^2$ (dof = 2)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCO</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>10</td>
<td>1.42</td>
<td>0.44</td>
</tr>
<tr>
<td>Control</td>
<td>85</td>
<td>110</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>87</td>
<td>116</td>
<td>31</td>
<td>234</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid abnormality</td>
<td>9</td>
<td>7</td>
<td>0</td>
<td>16</td>
<td>5.87</td>
<td>0.21</td>
</tr>
<tr>
<td>Control</td>
<td>85</td>
<td>110</td>
<td>29</td>
<td>224</td>
<td></td>
<td>0.18</td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>117</td>
<td>29</td>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulation abnormality</td>
<td>14</td>
<td>22</td>
<td>5</td>
<td>41</td>
<td>0.29</td>
<td>0.92</td>
</tr>
<tr>
<td>Control</td>
<td>85</td>
<td>110</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>99</td>
<td>132</td>
<td>34</td>
<td>265</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterine abnormality</td>
<td>13</td>
<td>16</td>
<td>2</td>
<td>31</td>
<td>1.10</td>
<td>0.69</td>
</tr>
<tr>
<td>Control</td>
<td>85</td>
<td>110</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>98</td>
<td>126</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.8 IL1B genotypes in recurrent miscarriage women with polycystic ovarian disease, thyroid abnormality, coagulation abnormality or uterine abnormality.
Table 4.13 The distribution of genotypes for IL1B polymorphism in women with unexplained recurrent miscarriage, recurrent miscarriage women in whom the cause is unknown or recurrent miscarriage women with retarded endometrium compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Total</th>
<th>$\chi^2$ (dof = 2)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unexplained RM</strong></td>
<td>30</td>
<td>48</td>
<td>7</td>
<td>85</td>
<td>1.94</td>
<td>0.40</td>
</tr>
<tr>
<td>Control</td>
<td>85</td>
<td>110</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>115</td>
<td>158</td>
<td>36</td>
<td>309</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Unknown cause</strong></td>
<td>9</td>
<td>15</td>
<td>1</td>
<td>25</td>
<td>2.10</td>
<td>0.44</td>
</tr>
<tr>
<td>Control</td>
<td>85</td>
<td>110</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>125</td>
<td>30</td>
<td>249</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Retarded endometrium</strong></td>
<td>6</td>
<td>18</td>
<td>3</td>
<td>27</td>
<td>3.16</td>
<td>0.19</td>
</tr>
<tr>
<td>Control</td>
<td>85</td>
<td>110</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>91</td>
<td>128</td>
<td>32</td>
<td>251</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.9 IL1B genotypes in recurrent miscarriage women with unexplained recurrent miscarriage, women whom the cause is unknown or recurrent miscarriage women with retarded endometrium.
Table 4.14 The distribution of genotypes for IL1B polymorphism in women with primary or secondary recurrent miscarriage compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Total</th>
<th>$\chi^2$ (dof = 2)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary miscarriage</td>
<td>36</td>
<td>65</td>
<td>7</td>
<td>108</td>
<td>4.93</td>
<td>0.09</td>
</tr>
<tr>
<td>Control</td>
<td>85</td>
<td>110</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>121</td>
<td>175</td>
<td>36</td>
<td>332</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary miscarriage</td>
<td>30</td>
<td>50</td>
<td>10</td>
<td>90</td>
<td>1.07</td>
<td>0.62</td>
</tr>
<tr>
<td>Control</td>
<td>85</td>
<td>110</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>115</td>
<td>160</td>
<td>39</td>
<td>314</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.10 IL1B genotypes in women with primary or secondary recurrent miscarriage.
Table 4.15 and 4.16 show the carriage rate of allele C and T, and the $\chi^2$ analysis results in the recurrent miscarriage women as a whole group or when divided to various different sub-groups according to cause of miscarriage in comparison with the control population. The results showed that there was no significant difference in the distribution of allele C and T carriage rates between controls and any group of recurrent miscarriage women.

Together, these results suggest that there was no difference in the frequency of the IL1B polymorphism in recurrent miscarriage women and the local control population.
Table 4.15 The carriage rates of allele C and allele T in the recurrent miscarriage women as a whole group or when divided into those suffering from primary and secondary recurrent miscarriage.

<table>
<thead>
<tr>
<th></th>
<th>With C (CC + CT)</th>
<th>Without C (TT)</th>
<th>Total</th>
<th>$\chi^2$ (dof = 1)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM group</td>
<td>186</td>
<td>20</td>
<td>206</td>
<td>1.11</td>
<td>0.36</td>
</tr>
<tr>
<td>Control</td>
<td>195</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>381</td>
<td>49</td>
<td>430</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary miscarriage</td>
<td>101</td>
<td>7</td>
<td>108</td>
<td>3.15</td>
<td>0.09</td>
</tr>
<tr>
<td>Control</td>
<td>195</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>296</td>
<td>36</td>
<td>332</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary miscarriage</td>
<td>80</td>
<td>10</td>
<td>90</td>
<td>0.20</td>
<td>0.71</td>
</tr>
<tr>
<td>Control</td>
<td>195</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>275</td>
<td>39</td>
<td>314</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.16 The carriage rates of allele C and allele T in the recurrent miscarriage women when divided to various different sub-groups depending on the cause of miscarriage.

<table>
<thead>
<tr>
<th></th>
<th>With C (CC + CT)</th>
<th>Without C (TT)</th>
<th>Total</th>
<th>$\chi^2$ (dof = 1)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine abnormality</td>
<td>29</td>
<td>2</td>
<td>31</td>
<td>1.10</td>
<td>0.40</td>
</tr>
<tr>
<td>Control</td>
<td>195</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>224</td>
<td>31</td>
<td>255</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unexplained</td>
<td>78</td>
<td>7</td>
<td>85</td>
<td>1.33</td>
<td>0.32</td>
</tr>
<tr>
<td>Control</td>
<td>195</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>273</td>
<td>36</td>
<td>309</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retarded endometrium</td>
<td>24</td>
<td>3</td>
<td>27</td>
<td>0.07</td>
<td>1.00</td>
</tr>
<tr>
<td>Control</td>
<td>195</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>219</td>
<td>32</td>
<td>251</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown cause</td>
<td>24</td>
<td>1</td>
<td>25</td>
<td>1.70</td>
<td>0.33</td>
</tr>
<tr>
<td>Control</td>
<td>195</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>219</td>
<td>30</td>
<td>249</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid abnormality</td>
<td>16</td>
<td>0</td>
<td>16</td>
<td>4.30</td>
<td>0.23</td>
</tr>
<tr>
<td>Control</td>
<td>195</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>211</td>
<td>29</td>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCO</td>
<td>8</td>
<td>2</td>
<td>10</td>
<td>0.41</td>
<td>0.63</td>
</tr>
<tr>
<td>Control</td>
<td>195</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>203</td>
<td>31</td>
<td>234</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulation</td>
<td>36</td>
<td>5</td>
<td>41</td>
<td>0.02</td>
<td>1.00</td>
</tr>
<tr>
<td>Control</td>
<td>195</td>
<td>29</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>231</td>
<td>34</td>
<td>265</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.3 Linkage disequilibrium studies of IL1RN and IL1B polymorphisms

Linkage disequilibrium analysis of the data obtained in this study was carried out using the 2LD computer programme to calculate the D' coefficient (a measure of the strength of linkage disequilibrium). The $\chi^2$ results showed that there was significant linkage disequilibrium between alleles of these two polymorphisms ($P = 3.19928 \times 10^{-5}$ for $D' = 0.28$ with a SD = 0.07). The $\chi^2$ analysis results showed that there is a strong association between allele 4 of IL1RN and allele C of IL1B.

4.3.4 Haplotyping studies of IL1RN and IL1B

Haplotyping distributions were then compared in various groups of recurrent miscarriage women to determine if a particular haplotype is over or under represented in either group. Haplotype analysis was carried out using the EH programme. To test whether haplotype frequencies are significantly different in cases and controls, the following formula was carried out using values produced by the EH programme: $T = \text{Ln}(L)\text{ cases} + \text{Ln}(L,\text{ controls}) - \text{Ln}(L,\text{ cases} + \text{controls})$

Table 4.17 shows haplotype distribution between alleles of IL1RN and IL1B in recurrent miscarriage women and controls. To compare haplotype distributions between recurrent miscarriage women and controls the following calculation was done:

$T = \text{Ln}(L,\text{ RM}) + \text{Ln}(L,\text{ controls}) - \text{Ln}(L,\text{ RM + controls})$

$= (-357.95) + (-407.8) - (-757.38)$

$= -8.37$

$\chi^2 = 2x T$ with 3 degree of freedom

$= 2x -8.37 = -16.74$, $P = 0.0008$

This showed that there was a significant differences in haplotype distributions in recurrent miscarriage women and controls.
Table 4.17 Haplotype distribution between alleles of IL1RN and IL1B in recurrent miscarriage women and controls.

<table>
<thead>
<tr>
<th>IL1B allele</th>
<th>IL1RN allele</th>
<th>Recurrent miscarriage</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 (4rpts)</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2 (2rpts)</td>
<td>0.11</td>
<td>0.10</td>
</tr>
<tr>
<td>1</td>
<td>3 (5rpts)</td>
<td>0.01</td>
<td>0.22</td>
</tr>
<tr>
<td>2</td>
<td>1 (4rpts)</td>
<td>0.24</td>
<td>0.15</td>
</tr>
<tr>
<td>2</td>
<td>2 (2rpts)</td>
<td>0.14</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>3 (5rpts)</td>
<td>0.0002</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Table 4.18 shows the haplotype distribution between alleles IL1RN and IL1B in women with primary and secondary recurrent miscarriage. To compare haplotype distributions between women with primary and secondary recurrent miscarriage the same formula was used:

\[ T = 1.18, \chi^2 = 2.36 \text{ with 5 degree of freedom, } P = 0.7974, \text{ not significant} \]

This showed that there was no significant differences in haplotype distributions in both groups of women.

Table 4.19 shows the haplotype distribution between alleles of IL1RN and IL1B in recurrent miscarriage women with and without PCOS. To compare haplotype distributions between recurrent miscarriage women with PCOS and recurrent miscarriage women without PCOS the same formula was also used.

\[ T = 3.8, \chi^2 = 7.6 \text{ with 5 degree of freedom, } P = 0.1797, \text{ not significant.} \]

The frequency of the IL1B allele 2/IL1RN*2 haplotype was increased compared with controls, but the sample number were too small for this to be significant.
Table 4.18 Haplotype distribution between alleles of IL1RN and IL1B in primary and secondary recurrent miscarriage women.

<table>
<thead>
<tr>
<th>IL1B allele</th>
<th>IL1RN allele</th>
<th>Primary miscarriage</th>
<th>Secondary miscarriage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 (4rpts)</td>
<td>0.52</td>
<td>0.48</td>
</tr>
<tr>
<td>1</td>
<td>2 (2rpts)</td>
<td>0.12</td>
<td>0.10</td>
</tr>
<tr>
<td>1</td>
<td>3 (5rpts)</td>
<td>0.00</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>1 (4rpts)</td>
<td>0.25</td>
<td>0.23</td>
</tr>
<tr>
<td>2</td>
<td>2 (2rpts)</td>
<td>0.12</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>3 (5rpts)</td>
<td>0.005</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table 4.19 Haplotype distribution between alleles of IL1RN and IL1B in recurrent miscarriage women with PCO and without PCO.

<table>
<thead>
<tr>
<th>IL1B allele</th>
<th>IL1RN allele</th>
<th>RM women with PCO</th>
<th>RM women without PCO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 (4rpts)</td>
<td>0.38</td>
<td>0.50</td>
</tr>
<tr>
<td>1</td>
<td>2 (2rpts)</td>
<td>0.12</td>
<td>0.11</td>
</tr>
<tr>
<td>1</td>
<td>3 (5rpts)</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>1 (4rpts)</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>2 (2rpts)</td>
<td>0.38</td>
<td>0.13</td>
</tr>
<tr>
<td>2</td>
<td>3 (5rpts)</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
4.3.5 Plasma IL-1β concentration measurement

Tables 4.20 and 4.21 shows the concentrations of IL-1β in the plasma of women with recurrent miscarriage divided according to their IL1RN and IL1B genotypes. Although there appeared to be a slightly higher plasma level of IL-1β in women with the 4, 2 IL1RN genotype than those with the 2, 2 genotype, this was not significant. Table 4.22 shows the concentrations of IL-1β in the same plasma of recurrent miscarriage women divided according to the different cause of miscarriage. Again, although there appeared to be some differences in plasma IL-1β levels in some groups of women, with retarded endometrium having low values and those with uterine abnormality having high values, the differences were not significant.
Table 4.20 The concentration of IL-1β in plasma of recurrent miscarriage women divided according to IL1RN genotype. Student t-test showed there was no significant difference in IL-1β levels in women with different IL1RN genotypes

<table>
<thead>
<tr>
<th>IL1RN genotype</th>
<th>Plasma IL-1β concentration (mean ± SEM) (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2 (n = 9)</td>
<td>0.26 ± 0.06</td>
</tr>
<tr>
<td>4,2 (n = 62)</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>4,4 (n = 84)</td>
<td>0.33 ± 0.06</td>
</tr>
</tbody>
</table>

Table 4.21 The concentration of IL-1β in plasma of recurrent miscarriage women divided according to IL1B genotype. Student t-test showed there was no significant difference in IL-1β levels in women with different IL1B genotypes

<table>
<thead>
<tr>
<th>IL1B genotype</th>
<th>Plasma IL-1β concentration (mean ± SEM) (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C,C (n = 52)</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>C,T (n = 87)</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td>T,T (n = 15)</td>
<td>0.48 ± 0.22</td>
</tr>
</tbody>
</table>
Table 4.22 The concentration of IL-1β in plasma of women with recurrent miscarriage divided according to different causes of miscarriage. Student t-test showed there was no significant difference in IL-1β levels in women according to different causes of miscarriage

<table>
<thead>
<tr>
<th>Cause</th>
<th>Plasma IL-1β concentration (mean± SEM) (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unexplained (n = 86)</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td>Retarded endometrium (n = 27)</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>Unknown (n = 25)</td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td>Thyroid (n = 16)</td>
<td>0.28 ± 0.05</td>
</tr>
<tr>
<td>PCO (n = 10)</td>
<td>0.26 ± 0.13</td>
</tr>
<tr>
<td>Uterine abnormality (n = 31)</td>
<td>0.44 ± 0.12</td>
</tr>
<tr>
<td>Coagulation (n = 41)</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>Primary miscarriage (n = 72)</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>Secondary miscarriage (n = 68)</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td>Whole group (n = 122)</td>
<td>0.44 ± 0.05</td>
</tr>
</tbody>
</table>
4.4 Discussion

In this study we investigated the distribution of genotypes, alleles and haplotypes of the IL1RN and IL1B genes in women with recurrent miscarriage and compared the results with that already known for a control Caucasian population (Blakemore et al., 1994; and unpublished data). Polymorphism of the IL1RN and IL1B genes may affect production of these molecules and may therefore affect maternal and foetal levels of these cytokines. This in turn could affect implantation and development of the feto-placental unit. Thus, the presence of different alleles may be associated with miscarriage.

4.4.1 IL1RN gene polymorphism

The results of this study demonstrate that there is no significant difference between the genotype or allele distribution of the IL1RN gene in controls and recurrent miscarriage women. This was true when the group was considered as a whole, and when the analysis was carried out on women divided according to the cause of the miscarriage except for women with PCOS. Comparison of the distribution of total alleles in women with recurrent miscarriage with the distribution of total alleles in controls, suggested a slightly higher allele frequency for allele 2, but this was not significant. A significant increase in the frequency of the allele 2 was seen in recurrent miscarriage women with PCOS. However, the numbers in this group are very small and a further study needs to be carried out on a larger group of PCOS women either with or without recurrent miscarriage.

The polymorphism of IL1RN is in intron two and therefore might not be expected to affect IL-1ra function. However, differences in this region may affect transcription of the IL-1ra gene and, therefore, the amount of protein produced. It has also been shown that IL1RN*2 (allele 2) is associated with increased secretion of IL-1β from cells cultured in vitro (Sanittila et al., 1998). Other studies have shown that the four-repeat allele is the most common allele of the IL1RN VNTR polymorphism (Tarlow et al., 1993).
The second most common allele in IL1RN gene in different populations is the two-repeat allele (IL1RN*2), and its presence has been associated with the severity of several inflammatory and autoimmune diseases, such as ulcerative colitis, multiple sclerosis, rheumatoid disease, psoriasis, alopecia areata and coronary heart disease (reviewed by Tazi-Ahnini et al., 2002). In addition, an association between IL1RN*2 and other diseases has been reported, particularly these associated with pro-inflammatory processes, such as systemic lupus erythematosus (SLE) (Blakemore et al., 1994).

In comparison to the results seen in this study, an association between IL1RN*2 and recurrent miscarriage has been reported recently among 105 Austrian women compared to 91 controls (Unfried et al., 2001). Another recent study reported a significantly increased frequency of IL1RN*3 (five-repeats) in women with recurrent miscarriage compared to a large control group of Finnish blood donors (Karhukorpi et al., 2003). However, in this study there were a significant number of ILRN*3 (5 repeats) genotypes both in the control population and recurrent miscarriage population, which was not seen in our study. Yet another study has shown no significant difference in the distribution of the IL1RN alleles in Caucasian recurrent miscarriage women and Caucasian control fertile women (Wang et al., 2002) and this would agree with the results of our study.

4.4.2 IL1B gene polymorphism
Analysis of the IL1B gene polymorphism demonstrated that there was no difference between the genotype or allele distribution in the control population and the genotype or allele distribution in women with recurrent miscarriage. This, again, was true whether the analysis was carried out either on data obtained from the group as a whole or when the women were divided according to the cause of miscarriage.

Hardy-Weinberg results showed that the IL1B genotype distributions were not as predicted for the allele frequencies in a population in Hardy-Weinberg equilibrium. There was an increased frequency of heterozygotes. Repeated PCR analysis showed that this was not due to incomplete digestion of the PCR products.
C to T base substitutions at position-511 and -31 of the promoter region of the IL1B gene have also been studied in recurrent miscarriage women and an increased frequency of the IL1B-511C and the IL1B-31T alleles was found in unexplained recurrent miscarriage women compared to controls (Helder et al., 2001). There was also an increased frequency of the IL1B-511C and IL1B-31T in recurrent miscarriage women with peripheral blood mononuclear cells which produced IFNγ in response to trophoblast antigen stimulation, compared to recurrent miscarriage women with peripheral blood mononuclear cells which did not produce IFNγ, suggesting an association with immune causes of recurrent miscarriage (Wang et al., 2002). However, a more recent study showed no evidence of an association between a polymorphism in the promoter region of the IL-1β gene (position-511) and recurrent miscarriage (Helder et al., 2002), which would agree with the results of our study.

4.4.3 Plasma IL-1β levels
When we analysed the amount of IL-1β in the plasma of blood samples from women with the different genotypes for IL1RN and IL1B-511, we found that there was no correlation between IL-1β plasma levels and either IL1B or IL1RN genotypes. These results are in agreement with another study which has reported no correlation between the IL1B polymorphism in exon 5 and IL-1β serum levels (Helder et al., 2001). It would therefore appear that plasma levels of IL-1β are not altered by the presence of different genotypes in these groups of women.

IL-1β serum levels vary during the menstrual cycle and are higher during the secretory phase (Cannon and Dinarello et al., 1985). The blood samples taken in our study were taken at different times in the cycle and this might obscure any differences due to genotype. In addition, it is levels of IL-1β in the fetoplacental unit that are likely to be most important in determining pregnancy outcome, rather than those in peripheral blood. A study comparing IL1B and IL1RN genotypes with endometrial or decidual IL-1β production would, perhaps, be more meaningful.
Recurrent miscarriage women may have elevated serum IL-1β levels during the first trimester of pregnancy compared to women with a normal pregnancy outcome (Shaarawy and Nagui, 1997). Although we were not able to compare plasma IL-1β levels in women with recurrent miscarriage and control women, we did compare levels of IL-1β in women with different causes of repeated pregnancy loss and there were no significant differences seen between any of the groups.

4.4.3 Haplotype analysis

Linkage disequilibrium analysis showed that there was a significant association between alleles of the IL1RN and IL1B polymorphisms; the common allele 4 in the IL1RN was inherited in combination with the common allele C in the IL1B. This is not unexpected since the genes are located close together. Linkage disequilibrium between IL-1 family genes has been reported previously (Cox et al., 1998).

The results of analysis of haplotype distribution indicated that there was no difference in haplotype distribution between controls and the whole group of recurrent miscarriage women, or between the various groups of women with different causes of miscarriage (PCOS & non PCOS, primary & secondary miscarriage).

Taken together, all these results suggest that the tandem repeat interleukin-1 receptor antagonist and the -511 interleukin-1B polymorphisms are not associated with recurrent miscarriage, but that the IL1RN polymorphism may be associated with PCOS.
Chapter 5

Expression of Interleukin-11 mRNA and protein in the endometrium of normal fertile women and women with recurrent miscarriage

5.1 Introduction

5.1.1 The interleukin-11

Interleukin-11 (IL-11) is a cytokine with pleiotropic functions in many tissues and cells (reviewed in Du and Williams, 1997). IL-11 was initially described as a growth factor, acting at multiple stages during haematopoiesis. It synergizes with other factors to stimulate progenitor cells and also stimulates cells of thrombopoietic, erythroid and lymphoid lineages (Du and Williams, 1994, 1997). IL-11 also has anti-inflammatory activity (Sands et al., 1999) and actions in bone remodelling (Maier et al., 1993), neuronal differentiation (Mehler et al., 1993), and female fertility in mice (Robb et al., 1998). It belongs to the gp130 family of cytokines, which includes interleukin-6 (IL-6), leukaemia inhibitory factor (LIF), oncostatin M, cardiotrophin-1 and ciliary neurotrophic factor (CNTF).

5.1.2 Interleukin-11 in endometrium

Recent studies from several laboratories have shown that IL-11 protein and mRNA is expressed in the human endometrium throughout the menstrual cycle (Dimtriadis et al., 2000; Cork et al., 2002; Chen et al., 2002; Karpovich et al., 2003). IL-11 protein is present at high levels in the epithelium and relatively low levels in the stroma. Expression of IL-11 in the stroma is increased in pre-decidualized stromal cells in the late secretory phase (Dimtriadis et al., 2000; Cork et al., 2001), when spontaneous decidual transformation of the stroma occurs around spiral arteries and in a compact layer below the surface epithelium.

IL-11 production by human endometrial stromal and epithelial cells in vitro is up-regulated by other cytokines such as tumour necrosis factor α (TNFα), transforming growth factor β (TGFβ) and interleukin-1α (IL-1α) (Cork et al.,
Stromal cell production of IL-11 is also increased by heparin binding epidermal growth factor (HB-EGF) (Karpovich et al., 2003). IL-11 has also been shown to affect human endometrial cell growth and function. It stimulates proliferative of endometrial stromal cells (Karpovich et al., 2003) and enhances progesterone-induced decidualization of endometrial stromal cells in vitro (Dimitriadis et al., 2002).

IL-11 would therefore appear to play an important role in endometrial function and implantation. The aim of this study was to compare endometrial IL-11 expression in control fertile women and recurrent miscarriage women during the mid-secretory phase of the cycle. We attempted to use both semi-quantitative mRNA analysis and to semi-quantify the immunostaining using H-scores. As these techniques had not been used in the lab before, we also used them to look at IL-11 expression throughout the cycle in normal fertile women.
5.2 Materials and Methods

5.2.1 Endometrial biopsies
Endometrial biopsies were obtained for this study from normal fertile women and recurrent miscarriage women as described in the Materials and Methods section. RT-PCR analysis of IL-11 mRNA expression was performed on endometrial biopsies obtained from 19 different normal fertile women during the early proliferative (n = 4), late proliferative (n = 3), early secretory (n = 3), mid secretory (n = 6) and late secretory (n = 3) phases of the menstrual cycle. Protein analysis was carried out by immunocytochemistry on only 17 of the same endometrial biopsy samples: 4 early proliferative, 3 late proliferative, 3 early secretory, 4 mid secretory and 3 late secretory. In a separate series of experiments IL-11 mRNA expression was compared in biopsies obtained from 9 normal fertile women and 17 recurrent miscarriage women during the mid-late secretory phase of the cycle. IL-11 protein expression was also compared in biopsies from 9 normal fertile women and 16 recurrent miscarriage women during the mid-secretory phase of the cycle.

Dating of the biopsies was calculated from the time of the last menstrual period. The approximate dating was confirmed by the morphological appearance after immunocytochemistry. Biopsies obtained between days 3-10 were termed early proliferative; days 10-14, late proliferative; days 15-19, early secretory; days 20-23, mid secretory and days 24-30, late secretory.

5.2.2 RT-PCR
RT-PCR analysis for expression of IL-11 mRNA in endometrial biopsies was carried out as described in chapter 2. Numerous attempts were made to optimise primer concentrations, annealing temperatures, number of cycles and MgCl₂ concentration. The final method is given in chapter 2. After optimization of the procedure, IL-11 mRNA expression was analysed by using IL-11 nested-PCR primers with GAPDH as an internal standard.
5.2.3 Immunocytochemistry

Cryostat sections were obtained from endometrial biopsies from normal fertile women and recurrent miscarriage women as described in the Materials and Methods section. All sections were stained according to the protocol described in the chapter 2. The protocol for each antibody was optimised, by using varying concentrations of both primary and secondary antibodies to identify the optimal combination for visualization of the antigen. The primary and secondary antibodies used in these experiments were obtained specifically for immunocytochemical studies. The primary antibody used was monoclonal mouse anti-human IL-11 (R+D Systems, Abingdon, U.K.) at a dilution of 1:100 in PBS containing 15μl blocking serum/ml and 5 drops biotin per ml and an incubation time of 24 hours at + 4°C. A parallel section was included, where the section was incubated with PBS containing 15μl blocking serum and 5 drops biotin, but no the primary antibody, as a negative control. The secondary antibody was a horse anti-mouse biotinylated antibody solution at a dilution of 1:200 in PBS containing 15μl blocking serum/ml (Vector Laboratories Inc. Burlingame, USA) and an incubation time of 30 minutes at room temperature. The binding was visualised using the ABC VECTOR kit and DAB substrate. All staining was assessed semi-quantitatively as described in chapter 2.
5.3 Results

5.3.1 Development of the PCR method to detect IL-11 mRNA expression

Tables 5.1, 5.2 and 5.3 show the 260/280 nm ratios and concentrations of mRNA extracted from cultured endometrial epithelial cells and biopsies from control fertile women and recurrent miscarriage women. mRNA extracted from primary cultures of endometrial epithelial cells was used to optimise the reaction. Extraction of mRNA from cells resulted in better quality mRNA than that obtained from tissue. This is indicated by the 260/280 nm absorbance ratios given in the tables. Ratios for mRNA extracted from cells were between 1.7 and 2.1 indicating good quality RNA. Ratios for mRNA extracted from biopsies ranged from 1.4 - 2.3. 11/17 biopsies from RM women resulted in 260/280 ratios of less than 1.7, compared with 2/19 biopsies from normal fertile women.

Firstly, an RT-PCR assay was performed on a house keeping gene, to check that mRNA had been converted to cDNA successfully. For this purpose, 7B6 primers were used which would amplify a gene coding for a non-histone protein in the cell, which is known to be produced abundantly.

Figure 5.1 shows the PCR products produced after amplification of mRNA extracted from 6 different endometrial biopsies using 7B6 primers. The expected band size is 434 base pairs. Distinct bands of the correct size were seen in the appropriate lanes, while no bands were seen in the no RT lanes. This shows that the mRNA samples have been converted to cDNA and can be amplified. Therefore, PCR amplification of mRNA using IL-11 primers was attempted.
Table 5.1 shows RNA concentration and ratios for RNA extracted from primary cultures of endometrial epithelial cells.

<table>
<thead>
<tr>
<th>Endometrial epithelial cells</th>
<th>RNA concentration</th>
<th>Ratio (260/280 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0 µg/µl</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>1.0 µg/µl</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>1.0 µg/µl</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>0.5 µg/µl</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>1.0 µg/µl</td>
<td>1.7</td>
</tr>
<tr>
<td>6</td>
<td>0.5 µg/µl</td>
<td>1.8</td>
</tr>
<tr>
<td>7</td>
<td>1.0 µg/µl</td>
<td>1.8</td>
</tr>
<tr>
<td>8</td>
<td>1.0 µg/µl</td>
<td>1.9</td>
</tr>
<tr>
<td>9</td>
<td>1.0 µg/µl</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>1.0 µg/µl</td>
<td>1.9</td>
</tr>
</tbody>
</table>
Table 5.2 shows RNA concentration and ratios for RNA extracted from 19 normal endometrial biopsy samples.

<table>
<thead>
<tr>
<th>Normal endometrium biopsies</th>
<th>RNA concentration</th>
<th>Ratio (260/280 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0 μg/μl</td>
<td>1.6</td>
</tr>
<tr>
<td>2</td>
<td>1.0 μg/μl</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>1.0 μg/μl</td>
<td>1.7</td>
</tr>
<tr>
<td>4</td>
<td>1.0 μg/μl</td>
<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td>1.0 μg/μl</td>
<td>1.9</td>
</tr>
<tr>
<td>6</td>
<td>1.0 μg/μl</td>
<td>1.8</td>
</tr>
<tr>
<td>7</td>
<td>1.0 μg/μl</td>
<td>1.8</td>
</tr>
<tr>
<td>8</td>
<td>1.0 μg/μl</td>
<td>1.9</td>
</tr>
<tr>
<td>9</td>
<td>1.0 μg/μl</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>1.0 μg/μl</td>
<td>1.9</td>
</tr>
<tr>
<td>11</td>
<td>1.0 μg/μl</td>
<td>2.0</td>
</tr>
<tr>
<td>12</td>
<td>1.0 μg/μl</td>
<td>1.9</td>
</tr>
<tr>
<td>13</td>
<td>1.0 μg/μl</td>
<td>2.3</td>
</tr>
<tr>
<td>14</td>
<td>2.5 μg/μl</td>
<td>2.0</td>
</tr>
<tr>
<td>15</td>
<td>1.0 μg/μl</td>
<td>1.9</td>
</tr>
<tr>
<td>16</td>
<td>1.0 μg/μl</td>
<td>1.8</td>
</tr>
<tr>
<td>17</td>
<td>1.0 μg/μl</td>
<td>1.9</td>
</tr>
<tr>
<td>18</td>
<td>0.4 μg/μl</td>
<td>2.1</td>
</tr>
<tr>
<td>19</td>
<td>1.0 μg/μl</td>
<td>1.9</td>
</tr>
</tbody>
</table>
Table 5.3 shows RNA concentration and ratios for RNA extracted from 17 recurrent miscarriage endometrial biopsy samples.

<table>
<thead>
<tr>
<th>Recurrent miscarriage endometrium biopsies</th>
<th>RNA concentration</th>
<th>Ratio (260/280 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0 μg/μl</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>1.5 μg/μl</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>1.0 μg/μl</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>1.0 μg/μl</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>1.0 μg/μl</td>
<td>1.4</td>
</tr>
<tr>
<td>6</td>
<td>1.0 μg/μl</td>
<td>1.6</td>
</tr>
<tr>
<td>7</td>
<td>1.0 μg/μl</td>
<td>1.5</td>
</tr>
<tr>
<td>8</td>
<td>1.0 μg/μl</td>
<td>1.6</td>
</tr>
<tr>
<td>9</td>
<td>1.1 μg/μl</td>
<td>1.4</td>
</tr>
<tr>
<td>10</td>
<td>1.0 μg/μl</td>
<td>1.8</td>
</tr>
<tr>
<td>11</td>
<td>1.0 μg/μl</td>
<td>2.1</td>
</tr>
<tr>
<td>12</td>
<td>10 μg/μl</td>
<td>2.1</td>
</tr>
<tr>
<td>13</td>
<td>7.7 μg/μl</td>
<td>2.1</td>
</tr>
<tr>
<td>14</td>
<td>1.5 μg/μl</td>
<td>1.6</td>
</tr>
<tr>
<td>15</td>
<td>2.3 μg/μl</td>
<td>1.6</td>
</tr>
<tr>
<td>16</td>
<td>2.7 μg/μl</td>
<td>1.6</td>
</tr>
<tr>
<td>17</td>
<td>2.0 μg/μl</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Figure 5.1 RT-PCR analysis of 7B6 from mRNA extracted from endometrial biopsies.

![Image of gel with markers and bands](image)

<table>
<thead>
<tr>
<th>Lane</th>
<th>Upper gel</th>
<th>Lower gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Marker (ΦX174 DNA/HaeIII)</td>
<td>Marker (ΦX174 DNA/HaeIII)</td>
</tr>
<tr>
<td>3</td>
<td>Endometrial biopsy 1</td>
<td>Endometrial biopsy 4</td>
</tr>
<tr>
<td>4</td>
<td>Negative sample (no RT)</td>
<td>Negative sample (no RT)</td>
</tr>
<tr>
<td>5</td>
<td>Endometrial biopsy 2</td>
<td>Endometrial biopsy 5</td>
</tr>
<tr>
<td>6</td>
<td>Negative sample (no RT)</td>
<td>Negative sample (no RT)</td>
</tr>
<tr>
<td>7</td>
<td>Endometrial biopsy 3</td>
<td>Endometrial biopsy 6</td>
</tr>
<tr>
<td>8</td>
<td>Negative sample (no RT)</td>
<td>Negative sample (no RT)</td>
</tr>
<tr>
<td>9</td>
<td>Negative sample (no RNA)</td>
<td>Negative sample (no RNA)</td>
</tr>
</tbody>
</table>
5.3.1.1 Optimisation of the PCR

The primer sequences used in the IL-11 PCR reaction were determined by searching the literature (Paul et al., 1990). The correctness of these sequences was then confirmed using the NCBI database. The entire coding sequence of IL-11 is 2281 bp long. These primers were designed to amplify a product of approximately 355 bp in length. Primer sequences are given in chapter 2.

a) MgCl₂ concentrations

First, the concentration of MgCl₂ was optimised. The IL-11 RT-PCR assay was performed on mRNA extracted from endometrial epithelial cells using a mastermix with 1.25mM, 1.5mM, 1.9mM, 2.5mM, and 3.75mM of MgCl₂ and annealing temperatures of 60°C and 58°C. The PCR reaction was repeated with each concentration of MgCl₂ and temperature, but no bands were seen indicating that amplification had not been successful (see figure 5.2).

b) Primer concentrations

The PCR assay was performed with all of the MgCl₂ concentrations previously used with annealing temperatures of 60°C and 58°C on genomic DNA extracted from blood samples to check whether the primers were working. The PCR assay was performed with different concentrations of each primer (forward & reverse). The concentrations were 0.12μM, 0.2μM, 0.3μM, 0.4μM, 0.6μM, 0.8μM, 1μM and 1.2μM. A strong band was seen of size 1778bp (the band size predicted for the PCR product from genomic DNA) with MgCl₂ concentrations of 1.5mM, primer concentrations of 0.12μM and an annealing temperature of 58°C (see figure 5.3).

The RT-PCR assay was then performed on RNA extracted from endometrial cells with MgCl₂ concentration of 1.5 mM, primer concentration of 0.12μM and 58°C as annealing temperature. A thin band of the correct size band (355bp) was seen in reaction lane 9 (see figure 5.4). However, no bands were seen with RNA extracted from biopsies or other epithelial cells.
Figure 5.2 An agarose gel to detect the products of the IL-11 RT-PCR reaction with mRNA extracted from epithelial cells with various concentrations of MgCl₂. No bands were seen with any of the MgCl₂ concentrations used.

<table>
<thead>
<tr>
<th>Lane</th>
<th>GEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker (ΦX174 DNA/HaeIII)</td>
</tr>
<tr>
<td>2</td>
<td>1.25 mM MgCl₂, 0.4μM F &amp; R primers</td>
</tr>
<tr>
<td>3</td>
<td>1.5 mM MgCl₂, 0.4μM F &amp; R primers</td>
</tr>
<tr>
<td>4</td>
<td>1.9 mM MgCl₂, 0.4μM F &amp; R primers</td>
</tr>
<tr>
<td>5</td>
<td>2.5 mM MgCl₂, 0.4μM F &amp; R primers</td>
</tr>
<tr>
<td>6</td>
<td>3.75 mM MgCl₂, 0.4μM F &amp; R primers</td>
</tr>
<tr>
<td>7</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 5.3 An agarose gel for the products produced after amplification of genomic DNA with IL-11 primers. Expected band size is 1778 bp.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker (ΦX174 DNA/HaeIII)</td>
</tr>
<tr>
<td>2</td>
<td>Sample 123 (1.5 mM MgCl₂, 0.12μM F &amp; R primers at 60°C, 30 cycles)</td>
</tr>
<tr>
<td>3</td>
<td>Sample 135 (1.5 mM MgCl₂, 0.12μM F &amp; R primers at 60°C, 30 cycles)</td>
</tr>
<tr>
<td>4</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Sample 123 (1.5 mM MgCl₂, 0.12μM F &amp; R primers at 58°C, 30 cycles)</td>
</tr>
<tr>
<td>6</td>
<td>Sample 135 (1.5 mM MgCl₂, 0.12μM F &amp; R primers at 58°C, 30 cycles)</td>
</tr>
<tr>
<td>7</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 5.4 Agarose gel showing the products of amplification of the IL-11 gene from RNA extracted from epithelial cells and endometrial biopsies. Expected product size is 355 bp. No bands were seen with RNA extracted from biopsies or epithelial cells, apart from amplification from one set of epithelial cells.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker (ΦX174 DNA/HaeIII)</td>
</tr>
<tr>
<td>2</td>
<td>Biopsy mRNA (2µl cDNA)</td>
</tr>
<tr>
<td>3</td>
<td>Epithelial cell mRNA (2µl cDNA)</td>
</tr>
<tr>
<td>4</td>
<td>Biopsy mRNA (3µl cDNA)</td>
</tr>
<tr>
<td>5</td>
<td>Epithelial cell mRNA (3µl cDNA)</td>
</tr>
<tr>
<td>6</td>
<td>Biopsy mRNA (4µl cDNA)</td>
</tr>
<tr>
<td>7</td>
<td>Epithelial cell mRNA (4µl cDNA)</td>
</tr>
<tr>
<td>8</td>
<td>Biopsy mRNA (5µl cDNA)</td>
</tr>
<tr>
<td>9</td>
<td>Epithelial cell mRNA (5µl cDNA)</td>
</tr>
<tr>
<td>10</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>
c) Numbers of the cycles
The PCR assay was repeated again with mRNA extracted from different endometrial epithelial cells. The thin band corresponding to the IL-11 RT-PCR product was only seen twice in numerous attempts. Finally, the effect of altering the number of cycles (30, 35, 60, 62, and 65) was investigated, but again the PCR product band was not consistently produced.

d) Nested PCR
As it become apparent that the straight RT-PCR reaction would not be able to detect IL-11 in endometrial biopsies, a nested RT-PCR reaction was attempted. The RT-PCR assay was performed with the same mastermix and 30 cycles. Specific nested primers were designed in-house by Dr. Alex Blakemore using OLIGO primer design software. The RT-PCR reaction was then repeated using the RT-PCR product of the first reaction as template with these new primers. A clear band of size 112bp was seen even after the reaction was carried out on mRNA extracted from endometrial biopsies (see figure 5.5). This is the size predicted for the product of the nested RT-PCR reaction.

5.3.2 Analysis of IL-11 mRNA in endometrial biopsies obtained from normal fertile women, throughout the menstrual cycle
The nested RT-PCR assay was then used on mRNA extracted from all the endometrial biopsies obtained from normal fertile women. The band sizes produced were compared with those obtained for GAPDH (as internal standard). Figure 5.5 shows that RT-PCR product could be obtained for IL-11 from mRNA prepared from normal endometrial biopsies collected throughout the menstrual cycle. However, bands for both IL-11 and GAPDH were only obtained from 14 of the 19 biopsies. The biopsy represented by lane 10 upper gel showed no IL-11 product, while biopsies represented by lanes 6, 7, 8, 11 upper gel and lane 4 lower gel showed no GAPDH products. Therefore, IL-11/GAPDH intensity ratios could only be calculated for 14 biopsies.
Figure 5.5 Agarose gel analysis of the RT-PCR products for both IL-11 (product bands 112 bp), and GAPDH (product bands 240 bp) from mRNA extracted from endometrial biopsies from normal fertile women.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Upper gel</th>
<th>Lower gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker(ΦX174DNA/HaeIII)</td>
<td>Marker(ΦX174DNA/HaeIII)</td>
</tr>
<tr>
<td>2</td>
<td>mRNA from biopsy 1</td>
<td>mRNA from biopsy 11</td>
</tr>
<tr>
<td>3</td>
<td>mRNA from biopsy 2</td>
<td>mRNA from biopsy 15</td>
</tr>
<tr>
<td>4</td>
<td>mRNA from biopsy 6</td>
<td>mRNA from biopsy 12</td>
</tr>
<tr>
<td>5</td>
<td>mRNA from biopsy 10</td>
<td>mRNA from biopsy 13</td>
</tr>
<tr>
<td>6</td>
<td>mRNA from biopsy 9</td>
<td>mRNA from biopsy 14</td>
</tr>
<tr>
<td>7</td>
<td>mRNA from biopsy 3</td>
<td>mRNA from biopsy 16</td>
</tr>
<tr>
<td>8</td>
<td>mRNA from biopsy 5</td>
<td>mRNA from biopsy 17</td>
</tr>
<tr>
<td>9</td>
<td>mRNA from biopsy 7</td>
<td>mRNA from biopsy 19</td>
</tr>
<tr>
<td>10</td>
<td>mRNA from biopsy 4</td>
<td>mRNA from biopsy 18</td>
</tr>
<tr>
<td>11</td>
<td>mRNA from biopsy 8</td>
<td>mRNA from biopsy 18</td>
</tr>
<tr>
<td>12</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Figure 5.6 shows the expression of the IL-11 mRNA in 14 normal endometrial biopsies obtained from different stages of the menstrual cycle compared to expression of GAPDH mRNA. IL11 mRNA expression was present in biopsies obtained throughout the menstrual cycle. The IL-11/ GAPDH intensity ratio was higher in biopsies obtained during the secretory phase (1.3 ± 0.2), compared to the proliferative phase (0.7 ± 0.2). The intensity ratio was lower in biopsies from the early proliferative phase (0.4 ± 0), and then increased throughout the cycle to reach a peak (1.4 ± 0.2) in the late secretory phase of the cycle. Intensity ratios were significantly higher (P < 0.05) for biopsies from the late secretory phase compared to those from the proliferative phase of the cycle.
Figure 5.6 Expression of IL-11 mRNA in endometrial biopsies obtained from normal fertile women throughout the cycle. Early proliferative (EP), late proliferative (LP), early secretory (ES), mid-secretory (MS) and late secretory (LS) phases of the menstrual cycle (values are mean ± SEM). * Significantly different to proliferative phase (P < 0.05).
5.3.3 Expression of IL-11 protein in human endometrium from normal fertile women

Figure 5.7 shows examples of IL-11 staining in sections obtained during the early proliferative (figure 5.7 a), late proliferative (figure 5.7 b), early secretory (figure 5.7 c), mid-secretory (figure 5.7 d) and late secretory (figure 5.7 e) phases of the menstrual cycle. A negative control is included, where a serial section had been incubated with PBS instead of the primary antibody (figure 5.7 f). The results show that, throughout the cycle, epithelial staining was more intense than that seen in the stromal cells of the endometrium. Glandular epithelial staining was weak in the early proliferative phase (figure 5.7 a), then increased in the late proliferative phase (figure 5.7 b), to reach a peak during the early-mid secretory phases (figure 5.7 c, d) of the menstrual cycle (at the time of implantation). In the late secretory phase (figure 5.7 e), the epithelial staining was decreased to a level similar to that seen in early proliferative endometrium. Stromal staining was very weak in early proliferative phase (figure 5.7 a) then increased slightly in the late proliferative phase (figure 5.7 b) to be moderate in the early secretory phase (figure 5.7 c) then peaking in the mid-secretory phase (figure 5.7 d) and remaining high in the late secretory phase (figure 5.7 e) of the menstrual cycle. Overall stromal staining showed greater intensity during the mid-late secretory phases of the cycle compared to the early proliferative phase.

Table 5.4 and figure 5.8 show the H-score values of IL-11 expression in epithelial and stromal cells from 17 biopsies obtained at different phases throughout the menstrual cycle. The H-score results suggested increased expression of IL-11 in epithelial cells (200-340) compared to the stromal cells (20-220). Statistical analysis showed significantly higher (P < 0.001) H-score IL-11 values for epithelial staining in biopsies obtained during the early-secretory phase of the cycle compared to epithelial H-scores in biopsies obtained in both the early proliferative and late secretory phases.
Figure 5.7 Expression of IL11 in endometrium of normal fertile women obtained in (a) early proliferative (b) late proliferative (c) early secretory (d) mid secretory and (e) late secretory phases of the cycle, (f) is a negative control. Magnification = X200
Table 5.4 H-score values for IL-11 expression in epithelial and stromal cells from 17 endometrial biopsies obtained at different times throughout the cycle. ** significantly different to EP and LS phases at \( P < 0.001 \), # significantly different to EP phase at \( P < 0.05 \), ## significantly different to EP phase at \( P < 0.01 \), ### significantly different to EP phase at \( P < 0.001 \).

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>Time in cycle</th>
<th>Epithelial H-score</th>
<th>Stromal H-score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Early proliferative</td>
<td>290</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Early proliferative</td>
<td>210</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>Early proliferative</td>
<td>240</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>Early proliferative</td>
<td>240 (245±17)</td>
<td>40 (50±17)</td>
</tr>
<tr>
<td>5</td>
<td>Late proliferative</td>
<td>300</td>
<td>120</td>
</tr>
<tr>
<td>6</td>
<td>Late proliferative</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>Late proliferative</td>
<td>320 (273±37)</td>
<td>220 (147±37)</td>
</tr>
<tr>
<td>8</td>
<td>Early secretory</td>
<td>340</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>Early secretory</td>
<td>330</td>
<td>180</td>
</tr>
<tr>
<td>10</td>
<td>Early secretory</td>
<td>330((333\pm3.3))***</td>
<td>100 (127±27)#</td>
</tr>
<tr>
<td>11</td>
<td>Mid-secretory</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>12</td>
<td>Mid-secretory</td>
<td>300</td>
<td>200</td>
</tr>
<tr>
<td>13</td>
<td>Mid-secretory</td>
<td>290</td>
<td>190</td>
</tr>
<tr>
<td>14</td>
<td>Mid-secretory</td>
<td>330 (280±28)</td>
<td>130 (180±17)###</td>
</tr>
<tr>
<td>15</td>
<td>Late secretory</td>
<td>240</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
<td>Late secretory</td>
<td>260</td>
<td>200</td>
</tr>
<tr>
<td>17</td>
<td>Late secretory</td>
<td>240 (247±6.6)</td>
<td>150 (150±29)##</td>
</tr>
</tbody>
</table>
Significantly higher H-scores for stromal IL-11 staining were seen in biopsies obtained from early-secretory (P < 0.05), mid-secretory (P < 0.001) and late-secretory (P < 0.01) phases of the cycle compared to H-scores for staining in early proliferative endometrium. The results overall suggest a different menstrual cycle pattern of expression of IL-11 in stromal and epithelial cells. In endometrial epithelium, a peak of IL-11 expression is seen in the early-mid secretory phases of the cycle, while stromal expression peaks during the mid-late secretory phases of the cycle.
Figure 5.8 Expression of IL-11 protein in human epithelial and stromal endometrium from normal fertile women. Values in graphs a and c are mean ± SEM. *** significantly different to early proliferative and late secretory values at P < 0.001, # significantly different to early proliferative values at P < 0.05, ## significantly different to early proliferative values at P < 0.01, ### significantly different to early proliferative values at P < 0.001
5.3.4 Endometrial IL-11 expression in biopsies from recurrent miscarriage women

5.3.4.1 Expression of IL-11 mRNA and GAPDH
Although 7B6 PCR products were obtained from mRNA extracted from biopsies obtained from recurrent miscarriage women, no IL-11 RT-PCR products could be obtained even using the nested PCR reaction.

5.3.4.2 Expression of IL-11 protein in biopsies from recurrent miscarriage women
Figure 5.9 shows IL-11 staining in sections obtained from recurrent miscarriage biopsies during the mid-secretory phase of the menstrual cycle (figure 5.9 c, d, e, f). Also shown is staining in endometrial sections obtained from normal fertile women at the same time in the cycle (figure 5.9 a, b). The pattern of staining in recurrent miscarriage sections showed very weak staining in the endometrial epithelial cells, compared to that seen in sections of biopsies from normal fertile women. In sections from biopsies obtained from a few RM women, stromal staining appeared stronger than that seen in normal fertile women, but overall the staining appeared very similar.

Table 5.5 and figure 5.10 show the H-score values of IL-11 expression in sections from biopsies from 16 recurrent miscarriage and 9 normal fertile women obtained during the mid-secretory phase of the menstrual cycle. Statistical analysis of the H-score results showed a decreased expression of epithelial cell IL-11 in endometrial biopsies from recurrent miscarriage women (201 ± 7.7) compared to that seen in the normal fertile women (286 ± 17) (P<0.01). However there were no significant differences in the H-score values obtained for IL-11 staining in the stromal compartment.
Figure 5.9  Expression of IL11 in endometrium of normal fertile (a) and (b) and recurrent miscarriage women (c), (d), (e) and (f) during the peri-implantation period. Magnification = X 200
Table 5.5  H-score values for endometrial epithelial and stromal IL-11 expression in 16 recurrent miscarriage biopsies and 9 normal biopsies obtained during the mid-secretory phase of the menstrual cycle, ** P < 0.01 compared to controls.

<table>
<thead>
<tr>
<th>Recurrent miscarriage biopsies</th>
<th>Epithelial staining</th>
<th>Stromal staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>160</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>280</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>250</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>250</td>
</tr>
<tr>
<td>5</td>
<td>210</td>
<td>250</td>
</tr>
<tr>
<td>6</td>
<td>190</td>
<td>230</td>
</tr>
<tr>
<td>7</td>
<td>210</td>
<td>200</td>
</tr>
<tr>
<td>8</td>
<td>200</td>
<td>150</td>
</tr>
<tr>
<td>9</td>
<td>180</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>11</td>
<td>150</td>
<td>170</td>
</tr>
<tr>
<td>12</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>13</td>
<td>180</td>
<td>150</td>
</tr>
<tr>
<td>14</td>
<td>250</td>
<td>220</td>
</tr>
<tr>
<td>15</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>16</td>
<td>200</td>
<td>(201 ± 7.7)**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Normal biopsies</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>220</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>230</td>
<td>160</td>
</tr>
<tr>
<td>3</td>
<td>350</td>
<td>140</td>
</tr>
<tr>
<td>4</td>
<td>350</td>
<td>200</td>
</tr>
<tr>
<td>5</td>
<td>270</td>
<td>200</td>
</tr>
<tr>
<td>6</td>
<td>300</td>
<td>280</td>
</tr>
<tr>
<td>7</td>
<td>260</td>
<td>230</td>
</tr>
<tr>
<td>8</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>340</td>
<td>(285 ± 17)</td>
</tr>
</tbody>
</table>
Figure 5.10  Expression of IL-11 protein in human epithelial and stromal endometrium in normal and recurrent miscarriage women. ** significantly different to normal endometrium P < 0.01
5.4 Discussion

5.4.1 IL-11 mRNA expression in biopsies obtained from normal fertile women

In this study we developed a method which enabled us to measure IL-11 mRNA expression in biopsies obtained from normal fertile women throughout the menstrual cycle. We attempted to semi-quantify the method by comparing amounts of mRNA with that produced from GAPDH. There is a possibility that in these experiments the accumulation of PCR products was not linear because 30 cycles was used in the RT-PCR, which may be too many to produce a linear relationship between the amount of mRNA present in the tissue and the product of the non-nested reaction. This should have been checked by carrying out the RT-PCR reaction with different amounts of cDNA. In addition, because of the low level of mRNA present, it was necessary to use a nested RT-PCR reaction which is also less quantitative than RT-PCR. However, the changes in IL-11 mRNA expression through the cycle seen in this study, are in agreement with changes seen in IL-11 protein expression and are similar to those reported by others (Dimitriadis et al., 2000). Our results showed that the IL-11/GAPDH mRNA intensity ratio was higher in biopsies obtained from the secretory phase than the proliferative phase. Previous studies have suggested that IL-11 plays a role in stromal cell decidualization (Dimitriadis et al., 2000; Dimitriadis et al., 2002). The increased in IL-11 expression in the endometrium at the very end of the cycle when decidualization takes place is consistent with it having a role in this process.

5.4.2 Expression of IL-11 protein in biopsies obtained from normal fertile women

In this study we investigated the expression of IL-11 protein in biopsies obtained from normal fertile women throughout the menstrual cycle. In contrast to RT-PCR, immunocytochemical analysis enabled the investigation of IL-11 expression in epithelial and stromal cells separately and was able to detect differences in IL-11 expression in epithelial and stromal compartment throughout the cycle. In agreement with the RT-PCR results for mRNA, these results showed that IL-11 protein was present in the human endometrium throughout the menstrual cycle, and was expressed by both stromal and epithelial cells, agreeing with the results from Dimitriadis et al. (2000) and Cork.
et al. (2001). H-score analysis of intensity of staining suggested that IL-11 protein expression was greater in epithelial cells than stromal cells throughout the menstrual cycle.

In the epithelial cells, maximum staining was seen in the early-mid secretory phase of the cycle, which corresponds to the time of implantation and is when there is maximum expression of other members of this family of cytokines, such as IL-6 and LIF in the endometrial epithelium (Laird et al., 1997, Von Wolff et al., 2002). In contrast to epithelial staining, the intensity of stromal IL-11 staining was increased during the mid-late secretory phases of the menstrual cycle. This increase in stromal IL-11 staining and decrease in epithelial IL-11 in the late secretory phase staining suggests that the increase in mRNA expression detected by RT-PCR at this time is due to stromal IL-11 expression. Previous studies in mice have suggested that IL-11 is only expressed by decidualized cells in the pregnant endometrium and not during the non-pregnant cycle (Bilinski et al., 1998; Robb et al., 1998; Davidson et al., 1997). Our results, and others (Dimitriadis et al., 2000; Cork et al., 2001) suggest that in human it is also expressed in the non-pregnant endometrium.

5.4.3 IL-11 mRNA expression in recurrent miscarriage women
In this study we attempted to use RT-PCR to investigate IL-11 mRNA expression in recurrent miscarriage women. Even using the nested RT-PCR reaction for IL11 no products could be obtained from the mRNA extracted from biopsies from recurrent miscarriage women. That may be due to the smaller size of the biopsies which were obtained as an out-patient procedure rather than from the operating theatre. The 260/280 nm absorbance ratios shown in table 5.3 suggest that the quality of mRNA extracted from biopsies from recurrent miscarriage women was less good than the mRNA extracted from cells or obtained from biopsies from normal fertile women, and this may be the reason why the RT-PCR analysis was unsuccessful. However, 7B6 products were obtained suggesting that the mRNA could be used for RT-PCR. The amounts of IL11 mRNA in the cell are likely to be less than that for 7B6 and therefore for successful amplification better quality mRNA may be needed.
5.4.4 Expression of IL-11 protein in recurrent miscarriage women

This study is one of the first to have investigated the expression of IL-11 protein in the endometrium of recurrent miscarriage women. H-score analysis results suggested that IL-11 expression was decreased in endometrial epithelial cells in the recurrent miscarriage women compared to that seen in normal fertile women. In contrast, stromal cell IL-11 expression showed no significant differences in expression in the recurrent miscarriage women and normal fertile women. The reduced expression of IL-11 in the endometrium of women with recurrent miscarriage is similar to that seen for other members of this family of cytokines (e.g. LIF and IL-6) and for IL-1 (Von Wolff et al., 2000; Cork et al., 1999).

In summary, our results confirm previous findings of increased expression of endometrial IL-11 in the mid- late secretory phase of the menstrual cycle and suggest decreased expression of endometrial epithelial cell IL-11 in recurrent miscarriage women.
Chapter 6

Expression of Interleukin-11 receptor α mRNA and protein in the endometrium of normal fertile women and women with recurrent miscarriage

6.1 Introduction

Work in IL-11Rα deficient transgenic mice provides strong evidence for a possible role for IL-11 in the decidual reaction, which is the response of the maternal endometrium essential for implantation and feto-placental development (Bilinski et al., 1998; Robb et al., 1998). Female mice lacking the IL-11Rα gene are infertile due to defective differentiation of the stroma in response to an implanting blastocyst; only small placentas developed leading to resorption of the embryo. Decidualization results in a change in morphology and functional activity of the stromal cells, characterised by an increased secretion of prolactin (Schatz et al., 1997). The IL-11 receptor comprises 2 sub-units IL-11Rα and gp130, which is the signalling molecule. Binding of IL-11 to IL-11Rα causes recruitment of gp130 and activation of intracellular pathways which probably include the Jak/ STAT and MAP kinase pathways.

Previous reports (Tabibzadeh et al., 1995; Cullinan et al., 1996; Dimitriadis et al., 2000; Chen et al., 2002) have shown that gp130 and IL-11Rα are expressed in the human endometrium throughout the menstrual cycle. Expression of both gp130 and IL-11Rα appears to be greater in epithelial cells than in stromal cells, but that there is little variation throughout the cycle (Cork et al., 2002; Dimitriadis et al., 2000).

The aims of this study were, therefore, to compare the expression of IL-11Rα mRNA and protein in the endometrium of normal fertile women and recurrent miscarriage women (RM) during the mid-secretory phase of the cycle. As for IL-11 we also looked at expression of IL-11Rα in endometrium from normal fertile women in order to verify the new techniques used.
6.2 Materials and Methods

6.2.1 Endometrial biopsies

Immunocytochemistry was used to determine the expression of IL-11Rα in sections taken from the same endometrial biopsies used in chapter 5. These were obtained from normal fertile women throughout the menstrual cycle and recurrent miscarriage women during the mid-secretory phase of the cycle. RT-PCR analysis of IL-11Rα mRNA expression was also performed on the same mRNA, extracted from endometrial biopsies obtained from normal fertile women and recurrent miscarriage women, used in chapter 5.

6.2.2 RT-PCR

RT-PCR analysis for expression of IL-11Rα mRNA in endometrial biopsies was carried out as described in chapter 2. IL-11Rα mRNA expression was demonstrated by reverse transcription of its mRNA to cDNA by an AMV reverse transcriptase mediated reaction, IL-11Rα RT-PCR reaction was then applied to mRNA extracted from the endometrial epithelial cells and then to endometrial biopsies. Once a method had been established, IL-11Rα mRNA expression was analysed using IL-11Rα nested primers and GAPDH as an internal standard.

6.2.3 Immunocytochemistry

Cryostat sections were obtained from endometrial biopsies as described in the Materials and Methods section. All sections were stained according to the protocol described in Chapter 2. The primary antibody used in these experiments was a goat anti-human IL-11Rα polyclonal antibody (R+D Systems, Abingdon, U.K.) at a concentration of 10μg/ml and an incubation time of 24 hours at + 4°C. A parallel section was included, where the section was incubated with PBS containing 15μl blocking serum and 5 drops biotin instead of the primary antibody, as a negative control. The secondary antibody was a rabbit anti-goat immunoglobulin biotinylated antibody solution at a dilution of 1:200 in PBS containing 15μl blocking serum/ml (Vector Laboratories, Inc. Burlingame, USA) and an incubation time of 30 minutes at room temperature. The binding was visualised using the ABC VECTOR kit and DAB substrate. All staining was assessed semi-quantitatively as described in Chapter 2.
6.3 Results

6.3.1 Analysis of IL-11Rα mRNA in endometrial biopsies obtained from normal fertile women throughout the menstrual cycle

A previous MSc student had designed the PCR primers and shown that an RT-PCR reaction with mRNA extracted from epithelial cells produced a band of the expected size (631 bp). However, application of this methodology to mRNA extracted from biopsies was harder; we faced problems in consistently obtaining PCR products from mRNA extracted from endometrial epithelial cells and biopsies every time a reaction was carried out.

Figure 6.1 shows the RT-PCR products produced using IL11Rα primers from mRNA obtained from endometrial epithelial cells. Lane 4 shows the two bands produced, one band of the expected size, 631 bp, and another of smaller size 251 bp. In order to check that these product bands corresponded to IL-11Rα sequence, three samples were prepared and sent for automatic sequencing (Figures 6.2 & 6.3). The sequence results which were obtained from the two bands were used in a BLAST search. The BLAST results showed that both sequences corresponded to that for IL-11Rα mRNA sequence. This suggests that there are two splice variants of IL-11Rα gene and these two products obtained contain a region of DNA that was shared by both the isoforms.

When the IL-11Rα RT-PCR reaction was initially applied to mRNA extracted from the endometrial biopsies, no bands were obtained. During the development of this method, a long "touch down" thermocycle methodology was attempted, with different annealing temperatures (see table 6.1). This resulted in some success, as illustrated in Figure 6.4, which shows RT-PCR products obtained from mRNA extracted from normal endometrial biopsies. Both the IL-11Rα RT-PCR products of 631 and 251 bp were seen. However, these PCR products could not be obtained from mRNA from all endometrial biopsies, so a nested RT-PCR reaction was used (nested primer sequences and methodology are given in Chapter 2).
Figure 6.5 shows products for the nested IL-11Rα RT-PCR reaction and the GAPDH RT-PCR reaction on mRNA obtained from normal endometrial biopsies, which were run together in the electrophoresis agarose gel. Lanes 2-11 in both the upper and lower gel show two bands, one for the IL-11Rα RT-PCR product of size 562 bp (upper band) and one for the GAPDH RT-PCR product of size 240 bp (lower band). From the electronic image produced from the gel, the band intensity ratios for IL-11Rα: GAPDH products were calculated.

Figure 6.6 shows the expression of the IL-11Rα mRNA in endometrial biopsies from normal fertile women obtained from different phases of the menstrual cycle compared to expression of GAPDH mRNA. Similarly to IL-11, expression of IL-11Rα was present in endometrium obtained throughout the menstrual cycle. The IL-11Rα/GAPDH intensity ratio was significantly higher (P < 0.05) for mRNA extracted from biopsies obtained in the late secretory phase of the cycle (1.5 ± 0.5) compared to those for mRNA extracted from biopsies obtained in the early proliferative phase of the cycle (0.3 ± 0.1).
Figure 6.1 Products produced from the RT-PCR analysis of mRNA from endometrial epithelial cells. Two products of sizes 631 bp and 251 bp were consistently seen.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker(ΦX174DNA/HaeIII)</td>
</tr>
<tr>
<td>2</td>
<td>Epithelial mRNA (RT)</td>
</tr>
<tr>
<td>3</td>
<td>Epithelial mRNA (RT)</td>
</tr>
<tr>
<td>4</td>
<td>Epithelial mRNA (RT)</td>
</tr>
<tr>
<td>5</td>
<td>Negative sample (no RT)</td>
</tr>
<tr>
<td>6</td>
<td>Negative sample (no RT)</td>
</tr>
<tr>
<td>7</td>
<td>Negative sample (no RT)</td>
</tr>
<tr>
<td>8</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Figure 6.2  The sequence results for IL-11Rα PCR product band of size 631 bp.
Figure 6.3 The sequence results for IL-11Ra PCR product band of size 251 bp.
Table 6.1 The "touch down" thermocycler programmes used for IL-11Rα RT-PCR reaction with mRNA extracted from endometrial biopsies.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Minute</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>65°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>72°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>94°C</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>62°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>72°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>94°C</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>59°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>72°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>94°C</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>56°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>72°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>94°C</td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>15</td>
<td>53°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>72°C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>72°C</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>4°C</td>
<td>Forever</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 6.4 Products produced from the analysis of mRNA extracted from endometrial biopsies from normal fertile using the IL11Rα primers. Two products of the same size as that seen for epithelial cells (631 & 251 bp) were seen.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker(ΦX174DNA/HaeIII))</td>
</tr>
<tr>
<td>2</td>
<td>Endometrial biopsy mRNA (RT)</td>
</tr>
<tr>
<td>3</td>
<td>Negative sample (no RT)</td>
</tr>
<tr>
<td>4</td>
<td>Endometrial biopsy mRNA (RT)</td>
</tr>
<tr>
<td>5</td>
<td>Negative sample (no RT)</td>
</tr>
<tr>
<td>6</td>
<td>Endometrial biopsy mRNA (RT)</td>
</tr>
<tr>
<td>7</td>
<td>Negative sample (no RT)</td>
</tr>
<tr>
<td>8</td>
<td>Endometrial biopsy mRNA (RT)</td>
</tr>
<tr>
<td>9</td>
<td>Negative sample (no RT)</td>
</tr>
<tr>
<td>10</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Figure 6.5 Products produced by RT-PCR from mRNA extracted from normal endometrial biopsies using IL-11Rα nested PCR and GAPDH primers.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Upper gel</th>
<th>Lower gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker(ΦX174DNA/Hae</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Endometrial biopsy 3</td>
<td>Endometrial biopsy 12</td>
</tr>
<tr>
<td>3</td>
<td>Endometrial biopsy 2</td>
<td>Endometrial biopsy 15</td>
</tr>
<tr>
<td>4</td>
<td>Endometrial biopsy 7</td>
<td>Endometrial biopsy 11</td>
</tr>
<tr>
<td>5</td>
<td>Endometrial biopsy 10</td>
<td>Endometrial biopsy 13</td>
</tr>
<tr>
<td>6</td>
<td>Endometrial biopsy 9</td>
<td>Endometrial biopsy 14</td>
</tr>
<tr>
<td>7</td>
<td>Endometrial biopsy 1</td>
<td>Endometrial biopsy 18</td>
</tr>
<tr>
<td>8</td>
<td>Endometrial biopsy 4</td>
<td>Endometrial biopsy 17</td>
</tr>
<tr>
<td>9</td>
<td>Endometrial biopsy 6</td>
<td>Endometrial biopsy 19</td>
</tr>
<tr>
<td>10</td>
<td>Endometrial biopsy 5</td>
<td>Endometrial biopsy 16</td>
</tr>
<tr>
<td>11</td>
<td>Endometrial biopsy 8</td>
<td>Endometrial biopsy 16</td>
</tr>
<tr>
<td>12</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Figure 6.6 Expression of IL-11Rα mRNA in endometrial biopsies from normal fertile women obtained throughout the cycle. Early proliferative (EP), late proliferative (LP), early secretory (ES), mid-secretory (MS) and late secretory (LS) phases of the menstrual cycle. Values in the second graph are mean ± SEM. * significantly different to early proliferative phase at P < 0.05.
6.3.2 Expression of IL11Rα protein in human endometrium from normal fertile women

Figure 6.7 shows examples of IL-11Rα staining in sections obtained during the early proliferative (figure 6.7 a), late proliferative (figure 6.7 b), early secretory (figure 6.7 c), mid-secretory (figure 6.7 d), and late secretory (figure 6.7 e) phases of the menstrual cycle. A negative control is included, where a serial section had been incubated with PBS instead of the primary antibody (figure 6.7 f). There is more staining present in the epithelial cells than stromal cells which suggest increased expression of IL11Rα in endometrial epithelial cells compared to endometrial stromal cells throughout the cycle. The staining intensity in both the epithelial and stromal compartments did not appear to alter considerably during the cycle. However, there did appear to be slightly increased staining in the epithelial cells in the mid-late secretory phases (figures 6.7 d, e) compared to the proliferative phase.

Table 6.2 and figure 6.8 show the H-score values of IL-11Rα expression in epithelial and stromal cells from 17 biopsies obtained at different phases throughout the menstrual cycle. The H-score results also suggested increased expression of IL-11Rα in epithelial cells (260-330) compared to the stromal cells (80-240). Statistical analysis showed that H-score values for staining intensity in epithelial cells in biopsies from the late secretory phase of the cycle were significantly greater than for biopsies obtained during the early proliferative (P < 0.05), late proliferative (P < 0.05) and mid-secretory (P < 0.05) phases of the cycle. Although the H-score values for stromal staining also suggested increased expression in the mid-late secretory phases of the menstrual cycle, these values were not significantly different, partly because of the large variation in staining intensities seen.
Figure 6.7 Expression of IL11 receptor alpha in endometrium of normal fertile women obtained in a) early proliferative b) late proliferative c) early secretory d) mid secretory and e) late secretory stages, f is a negative control. Magnification = X 200
Table 6.2 H-score values for IL-11Rα expression in endometrial epithelial and stromal cells from 17 biopsies obtained at different times throughout the cycle. * significantly greater than early proliferative, late proliferative and mid secretory endometrium at P < 0.05.

<table>
<thead>
<tr>
<th>Biopsies</th>
<th>Time in cycle</th>
<th>Epithelial staining</th>
<th>Stromal staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Early proliferative</td>
<td>310</td>
<td>230</td>
</tr>
<tr>
<td>2</td>
<td>Early proliferative</td>
<td>290</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>Early proliferative</td>
<td>290</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
<td>Early proliferative</td>
<td>270 (290 ± 8.2)</td>
<td>80 (178 ± 33)</td>
</tr>
<tr>
<td>5</td>
<td>Late proliferative</td>
<td>290</td>
<td>230</td>
</tr>
<tr>
<td>6</td>
<td>Late proliferative</td>
<td>290</td>
<td>180</td>
</tr>
<tr>
<td>7</td>
<td>Late proliferative</td>
<td>290 (290 ± 0)</td>
<td>230 (213 ± 17)</td>
</tr>
<tr>
<td>8</td>
<td>Early secretory</td>
<td>290</td>
<td>240</td>
</tr>
<tr>
<td>9</td>
<td>Early secretory</td>
<td>260</td>
<td>230</td>
</tr>
<tr>
<td>10</td>
<td>Early secretory</td>
<td>300 (283 ± 12)</td>
<td>120 (200 ± 35)</td>
</tr>
<tr>
<td>11</td>
<td>Mid-secretory</td>
<td>300</td>
<td>230</td>
</tr>
<tr>
<td>12</td>
<td>Mid-secretory</td>
<td>270</td>
<td>140</td>
</tr>
<tr>
<td>13</td>
<td>Mid-secretory</td>
<td>290</td>
<td>220</td>
</tr>
<tr>
<td>14</td>
<td>Mid-secretory</td>
<td>280 (285 ± 6)</td>
<td>230 (205 ± 22)</td>
</tr>
<tr>
<td>15</td>
<td>Late secretory</td>
<td>330</td>
<td>240</td>
</tr>
<tr>
<td>16</td>
<td>Late secretory</td>
<td>330</td>
<td>230</td>
</tr>
<tr>
<td>17</td>
<td>Late secretory</td>
<td>300 (320 ± 10)*</td>
<td>240 (237 ± 3.3)</td>
</tr>
</tbody>
</table>
Figure 6.8 Expression of IL11Rα protein in human epithelial and stromal cells in normal endometrial biopsies. Early proliferative (EP), late proliferative (LP), early secretory (ES), mid-secretory (MS) and late secretory (LS) phases of the menstrual cycle. Values in graphs a and c are mean ± SEM. * significantly different to EP, LP and mid-secretory endometrium at P < 0.05.
6.3.3 Endometrial IL11Rα expression in biopsies from recurrent miscarriage women

6.3.3.1 Expression of IL11Rα mRNA and GAPDH

Our previous studies on IL-11 mRNA expression showed that 7B6 PCR products were successfully obtained from mRNA extracted from the 17 biopsies obtained from recurrent miscarriage women. However, even using the nested PCR reaction, IL-11Rα products were only obtained from mRNA extracted from endometrial biopsies from 10 recurrent miscarriage women.

Figure 6.9 shows the products of the nested RT-PCR for the IL11Rα and the RT-PCR for GAPDH mRNA extracted from endometrial biopsies from recurrent miscarriage women. Bands for both IL-11Rα and GAPDH were obtained from all 10 biopsies. The electronic image produced from this gel was used to calculate the band intensity ratios for IL11Rα: GAPDH products.

Figure 6.10 shows the IL-11Rα/GAPDH intensity ratios in biopsies from 10 recurrent miscarriage and 6 normal women obtained during the mid-secretory phase of the cycle. There were no significant differences in these ratio values for biopsies obtained from normal fertile women (0.8 ± 0.3) and recurrent miscarriage women (1.2 ± 0.3) suggesting that there are no differences in IL-11Rα mRNA expression in these two groups of women.
Figure 6.9 Products produced by RT-PCR from mRNA extracted from biopsies from 10 recurrent miscarriage women using IL-11Rα & GAPDH primers.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker(ΦX174DNA/HaeIII)</td>
</tr>
<tr>
<td>2</td>
<td>Endometrial biopsy 2 (Ratio: 1.8 nm)</td>
</tr>
<tr>
<td>3</td>
<td>Endometrial biopsy 10 (ratio:: 1.8 nm)</td>
</tr>
<tr>
<td>4</td>
<td>Endometrial biopsy 11 (ratio:: 2.1 nm)</td>
</tr>
<tr>
<td>5</td>
<td>Endometrial biopsy 12 (ratio:: 2.1 nm)</td>
</tr>
<tr>
<td>6</td>
<td>Endometrial biopsy 13 (ratio:: 2.1 nm)</td>
</tr>
<tr>
<td>7</td>
<td>Endometrial biopsy 6  (ratio:: 1.6 nm)</td>
</tr>
<tr>
<td>8</td>
<td>Endometrial biopsy 14 (ratio: 1.6 nm)</td>
</tr>
<tr>
<td>9</td>
<td>Endometrial biopsy 15 (ratio: 1.6 nm)</td>
</tr>
<tr>
<td>10</td>
<td>Endometrial biopsy 16 (ratio: 1.6 nm)</td>
</tr>
<tr>
<td>11</td>
<td>Endometrial biopsy 8  (ratio: 1.6 nm)</td>
</tr>
<tr>
<td>12</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Figure 6.10 Expression of IL11Rα mRNA in endometrial biopsies from 10 women with recurrent miscarriage and 6 control women
6.3.3.2 Expression of IL-11Rα protein in biopsies from recurrent miscarriage women

Figure 6.11 shows IL-11Rα staining in sections from biopsies obtained from recurrent miscarriage women during the mid-secretory (figure 6.11 c, d and e) phase of the menstrual cycle. Also shown is staining in sections obtained from normal fertile women (figure 6.11 a, b) and the negative control (figure 6.11 f). The pattern of staining for both stromal cells and epithelial cells was similar in sections of endometrium from both normal fertile and recurrent miscarriage women. Epithelial endometrial cells showed more staining than that seen in the stromal endometrial cells in both recurrent miscarriage women and normal fertile women.

Table 6.3 and figure 6.12 show the H-score values of IL-11Rα expression in sections from biopsies from 16 recurrent miscarriage and 9 control women obtained during the mid-secretory phase of the menstrual cycle. Statistical analysis of the H-score results also showed no significant difference in the expression of epithelial cell IL-11Rα in recurrent miscarriage women (329 ± 4) compared to that seen in the normal fertile women (321 ± 7). There were also no differences in H-score values for IL-11Rα stromal cell staining in recurrent miscarriage women (254 ± 5.5) compared to that seen in the normal fertile women (248 ± 10).
Figure 6.11 Expression of IL11 receptor alpha in endometrium from normal fertile women (a and b) and recurrent miscarriage women (c, d, and e) during the peri-implantation period, f is a negative control. Magnification = X 200
<table>
<thead>
<tr>
<th>Recurrent miscarriage biopsies</th>
<th>Epithelial staining</th>
<th>Stromal staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>340</td>
<td>260</td>
</tr>
<tr>
<td>2</td>
<td>340</td>
<td>270</td>
</tr>
<tr>
<td>3</td>
<td>330</td>
<td>250</td>
</tr>
<tr>
<td>4</td>
<td>330</td>
<td>260</td>
</tr>
<tr>
<td>5</td>
<td>320</td>
<td>230</td>
</tr>
<tr>
<td>6</td>
<td>330</td>
<td>270</td>
</tr>
<tr>
<td>7</td>
<td>320</td>
<td>250</td>
</tr>
<tr>
<td>8</td>
<td>300</td>
<td>250</td>
</tr>
<tr>
<td>9</td>
<td>320</td>
<td>280</td>
</tr>
<tr>
<td>10</td>
<td>350</td>
<td>280</td>
</tr>
<tr>
<td>11</td>
<td>320</td>
<td>250</td>
</tr>
<tr>
<td>12</td>
<td>330</td>
<td>280</td>
</tr>
<tr>
<td>13</td>
<td>320</td>
<td>200</td>
</tr>
<tr>
<td>14</td>
<td>300</td>
<td>250</td>
</tr>
<tr>
<td>15</td>
<td>360</td>
<td>220</td>
</tr>
<tr>
<td>16</td>
<td>350</td>
<td>260 (329 ± 4)</td>
</tr>
<tr>
<td>Normal biopsies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>330</td>
<td>280</td>
</tr>
<tr>
<td>2</td>
<td>330</td>
<td>260</td>
</tr>
<tr>
<td>3</td>
<td>280</td>
<td>190</td>
</tr>
<tr>
<td>4</td>
<td>310</td>
<td>230</td>
</tr>
<tr>
<td>5</td>
<td>350</td>
<td>240</td>
</tr>
<tr>
<td>6</td>
<td>340</td>
<td>290</td>
</tr>
<tr>
<td>7</td>
<td>320</td>
<td>250</td>
</tr>
<tr>
<td>8</td>
<td>330</td>
<td>250</td>
</tr>
<tr>
<td>9</td>
<td>300 (321 ± 7)</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(248 ± 10)</td>
</tr>
</tbody>
</table>
Figure 6.12 Expression of IL-11Rα protein in human epithelial and stromal endometrium obtained from normal and recurrent miscarriage women during the mid-secretory phase of the cycle
6.4 Discussion

6.4.1 IL-11Rα mRNA expression in human endometrium

In this study we investigated IL-11Rα mRNA expression in human endometrium throughout the menstrual cycle. Our results demonstrated the presence of IL-11Rα mRNA in the human endometrium. As for the RT-PCR results for IL-11, these results for mRNA analysis for IL-11Rα need to be considered with caution. Again there were very low levels of IL-11R mRNA present, which meant that a large number of cycles was required to be used in the RT-PCR and that a nested PCR reaction was used. However, again the IL-11Rα mRNA results are agreement with IL-11Rα protein results, suggesting that they may reflect mRNA levels in vivo. Our results are also in agreement with previous reports (Dimitriadis et al., 2000, Chen et al., 2002, Karpovich et al., 2003), which have shown that IL-11Rα mRNA is expressed in the human endometrium. In our study the IL-11Rα/ GAPDH intensity ratio was significantly higher in the late secretory phase compared to the proliferative phase. This is in contrast to previous studies (Dimitriadis et al., 2000, Chen et al., 2002, Karpovich et al., 2003), which have reported that IL-11Rα mRNA levels are constant throughout the menstrual cycle. Increasing levels of IL-11Rα mRNA during the late secretory phase of the cycle would suggest a possible function for IL-11 in human decidualization, similar to its role in mice (Dimitriadis et al., 2000).

6.4.2 Expression of IL11Rα protein in human endometrium from normal fertile women

In this study, we investigated the expression of IL-11Rα protein in the human endometrium throughout the menstrual cycle. Immunocytochemical analysis allows discrimination between epithelial and stromal IL-11Rα expression and showed that it was expressed by both stromal and epithelial cells. This is in agreement with previous studies (Dimitriadis et al., 2000, Cork et al., 2001). H-score analysis of intensity of staining suggested increased expression of IL-11Rα protein in endometrial epithelial cells compared to the endometrial stromal cells throughout the menstrual cycle, agreeing with the results from Cork et al. (2002).
In the late secretory phase of the menstrual cycle, there was a slight increase in staining intensity for IL-11Rα in both epithelial and stromal cells compare to that seen in the proliferative phase of the menstrual cycle. This slight increase in both epithelial and stromal IL-11Rα expression is similar to that reported previously in non-quantitative studies (Cork et al., 2002).

The fact that increases in both IL-11Rα mRNA and protein were seen in late secretory endometrium provides strong evidence for increased expression of IL-11Rα in the endometrium of this time. The high levels of IL-11Rα protein in the human endometrium during the late secretory phase shown in this study and in previous studies, suggests that IL-11 is involved in the functional differentiation that occurs during decidualization of human endometrial stromal cells.

6.4.3 IL-11Rα mRNA expression in recurrent miscarriage women
This study is one of the first to investigate the presence of IL-11Rα mRNA in the human endometrium of women with recurrent miscarriage and compare it to expression in normal fertile women. RT-PCR results confirmed the expression of IL-11Rα mRNA in biopsies obtained from recurrent miscarriage women during the mid-secretory phase of the cycle. Our results revealed no significant difference in the expression of IL-11Rα mRNA between biopsies obtained from normal fertile women and recurrent miscarriage women during the peri-implantation period.

6.4.4 Expression of IL-11Rα protein in recurrent miscarriage women
This study is also one of the first studies that have investigated the expression of IL-11Rα protein in the recurrent miscarriage women. As in biopsies from normal fertile women, IL-11Rα protein expression was higher in epithelial cells compared to stromal cells. In agreement with the expression of IL-11Rα mRNA, results on protein expression also suggest that there are no significant differences in the expression of IL-11Rα protein in either stromal or epithelial cells in biopsies obtained from normal fertile women or recurrent miscarriage women. Thus, it would appear that, in contrast to IL-11, there are no significant differences in expression of IL-11Rα in the endometrium of normal fertile women and women with recurrent miscarriage. The results in normal fertile
women suggest that there is maximal expression of endometrial IL-11Rα in the late secretory phase of the cycle. Therefore, it might be better to compare endometrial IL-11Rα expression at this time in the cycle, rather than in the mid-secretory phase. In addition, it appears that IL-11Rα plays a role in decidualization and therefore this could be the time of maximal biological importance for its expression.
Chapter 7

General Discussion

7.1 Introduction

This study has investigated the possible role of some pro-inflammatory cytokines and leptin in recurrent miscarriage. It has concentrated on the pro-inflammatory cytokines, IL-1 (IL-1α, IL-1β), IL-11, IL-11Rα and leptin, as previous studies have suggested that these are important in endometrial function, particularly decidualization and, successful implantation. The techniques used were PCR analysis of IL1RN, IL1B and leptin receptor polymorphisms in DNA extracted from peripheral blood samples and RT-PCR and immunocytochemical analysis of IL-11Rα and IL-11 mRNA and protein in endometrial biopsies. PCR analysis was used for genotyping, while RT-PCR results shows expression of mRNA, which confirms synthesis, rather than presence of cytokines in the tissue. Immunocytochemistry has the advantage of enabling detection of cytokine protein in the human endometrial biopsies, and determining which cell populations are involved in their production.

In the first part of the study, polymorphisms of IL1B, IL1RN and the leptin receptor genes were investigated in a large population of recurrent miscarriage women (n = 206) and genotype distributions compared with those previously determined for a control population. In the recurrent miscarriage group of women, the causes of miscarriage had been extensively investigated and were known for the majority of women. This allow us to compare genotype distribution in groups of recurrent miscarriage women with different causes. In the case of the IL1RN polymorphism, this suggested differences in women with PCO compared to controls, although no differences were seen in the whole recurrent miscarriage group compared to controls. In the second part of the study, levels of expression of IL-11 and IL-11Rα in endometrium of recurrent miscarriage women and control women were compared using RT-PCR and immunocytochemistry. The reasons for this change in direction of study was; (1) very few significant results were found in the polymorphisms studies and (2) our previous work and that of others, suggested the importance of endometrial IL-11 in implantation and the prevention of pregnancy loss in mice.
The major results from these studies suggest a possible functional role for the leptin receptor in preventing miscarriage in some groups of recurrent miscarriage women. However, no differences in frequency or carriage of the tandem repeat polymorphisms of the IL1RN gene and the C to T polymorphisms at position -511 of the IL1B gene were seen in recurrent miscarriage women compared to controls. This may suggest either that these polymorphisms do not affect IL-1 levels, or that IL-1 does not play a major role in recurrent miscarriage. We have also confirmed previous findings of increased expression of endometrial IL-11 and IL-11Ra in the mid-late secretory phase of the cycle and shown decreased expression of endometrial epithelial IL-11 but not IL-11Ra in recurrent miscarriage women.

7.2 GLN223ARG leptin receptor gene polymorphism in recurrent miscarriage women

The results from the GLN223ARG leptin receptor gene polymorphism study demonstrated that although the AA genotype was more common in women with recurrent miscarriage than in the general population, there was no significant difference between genotype distribution in controls and women with recurrent miscarriage as a whole group. However, when divided according to the cause of recurrent miscarriage there was a significant increase in the frequency of the AA genotype in women with secondary recurrent miscarriage and those whose aetiology is unknown. The increase in AA genotype in these groups was associated with higher allele A frequency and carriage rate. A reason for the differences seen in women with secondary miscarriage (not primary miscarriage) is difficult to determine, but it may be due to alterations in the endometrium or decidua as a result of pregnancy. It is also difficult to understand why differences were seen in women whose aetiology is unknown because in this group of women, not all the investigations were completed.

The GLN223ARG leptin receptor polymorphism investigated in this study results in the substitution of amino acids in the transmembrane section of the receptor which may affect the function of that receptor. Recent studies have reported an association in GLN223ARG leptin receptor polymorphism and leptin levels, fat mass and body mass index (BMI) in a population of Caucasian normal
postmenopausal women and middle-aged men (Quinton et al., 1998; Chagnon et al., 2000). Another study has reported lower leptin concentrations in women with recurrent miscarriage compared with normal fertile women (Laird et al., 2001). Leptin also appears to be an important regulator of foetal growth as many studies have shown that low concentrations of maternal plasma leptin are associated with sub-optimal pregnancy outcomes (Lage et al., 1999; Lea et al., 2000; Laird et al., 2001).

Leptin is known to interact with cytokine networks (Granowitz, 1997; Yamaguchi et al., 1998). Leptin is regulated in several tissues by interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) (Sarraf et al., 1997), and leptin may itself induce the synthesis of inflammatory cytokines in vivo and in vitro (Loffreda et al., 1998). Thus, leptin may affect pregnancy outcome by affecting the balance of cytokines in the foetal-placental unit or contribute to the development of placental vessels, together with other growth factors such as vascular endothelial growth factor (Shiraishi et al., 1996; Mühlhauser et al., 1996).

Although, the results of the present study suggest that expression of the allele A may be associated with recurrent miscarriage, a further large study is required to confirm the findings.

7.3 IL-1RN and IL-1B gene polymorphisms in RM women

Overall the results from these studies were disappointing in that very few significant differences were seen in genotype distribution or allele frequency in recurrent miscarriage women compared to controls. An increased frequency of 2,2 genotype for the IL1RN gene polymorphism was seen in recurrent miscarriage women with PCOS. However, the numbers in this group were very small, and a further study needs to be carried out with larger numbers of recurrent miscarriage women with PCOS and/or women with PCOS without recurrent miscarriage.
The polymorphism of IL1RN is in intron two and therefore might not affect IL-1ra function. However, previous reports have suggested that differences in this region may affect transcription of the IL-1ra gene and the amount of protein produced (Blakemore et al., 1994; Unfried et al., 2001). In agreement with the results of our study, Wang et al. (2002) has shown no significant difference in the distribution of the IL1RN alleles in recurrent miscarriage women and control fertile women. However, other studies have seen associations between this polymorphism and recurrent miscarriage. One study has shown an increased frequency of IL1RN*2 and recurrent miscarriage (Unfried et al., 2001). While another has reported a significant increased frequency of IL1RN*3 (five-repeats) in 37 Finnish women with recurrent miscarriage compared to 800 controls (Karhukorpi et al., 2003).

No differences were seen in the genotype distributions or allele frequencies for the -511 polymorphism in the promoter region of the IL1B gene in recurrent miscarriage women and controls. Others have also studied C to T base substitutions at positions -511 and -31 of the promoter region of the IL1B gene in women with recurrent miscarriage. One study has shown no significant association between the IL1B-511 polymorphisms and recurrent miscarriage (Helfer et al., 2002) and this agrees with our results. In contrast, a recent study has shown an increased frequency of the IL1B-511C allele in women with unexplained recurrent miscarriage compared to controls (Wang et al., 2002). The reason for these differences is not clear, but is common in allele association studies and is probably due to selection criteria for patient groups and controls (ascertainment bias), different populations, different selection of controls (ethnically-matched, admixture, inbreeding, etc.), and power of the study (the number of subjects).

Measurement of plasma IL-1β concentrations in this group of recurrent miscarriage women showed that there was no correlation between IL-1β plasma levels and either IL1B or IL1RN genotypes, and these are in agreement with a recent study which has reported no correlation between the IL1B-511 polymorphism and IL-1β serum levels (Helfer et al., 2001). It would, therefore, appear that the presence of different alleles of this polymorphisms do not affect plasma IL-1β levels in recurrent miscarriage women. However, it is possible,
that although there were no differences in IL1B-511 genotype distribution in recurrent miscarriage women and the control population, IL-1β may still play a role in recurrent miscarriage. In addition, the levels of IL-1β (and IL-1ra) at the materno-feto interface are likely to be more important than levels in the plasma at determining pregnancy outcome.

Linkage disequilibrium analysis indicated that there was a significant association between alleles of the IL1RN and IL1B polymorphisms. This is expected because the genes are located close together on chromosome 2. A previous study has also reported linkage disequilibrium between IL-1 family genes (Cox et al., 1998). The results of analysis of haplotype distribution indicated that there was no difference in haplotype distribution between controls and the whole group of recurrent miscarriage women, or between groups of women with different causes of recurrent miscarriage (PCO & non PCO, primary & secondary miscarriage).

7.4 Interactions between IL-1 and leptin
Recent studies have suggested that the leptin and IL-1 systems might interact in the control of human embryo implantation, and it has been suggested that the IL-1 system could play an important role in the cross-talk established between the preimplantation embryo and the receptive endometrium during the early phase of human implantation process (Simon et al., 1994). Leptin promotes preimplantation embryo development (Kawamura et al., 2002), leptin (Gonzalez et al., 2000b; Wu et al., 2002) and leptin receptors (OB-R) are expressed by the endometrium (Alfer et al., 2000; Gonzalez et al., 2000b; Kitawaki et al., 2000; Wu et al., 2002; Quinton et al., 2003).

IL-1 up-regulates endometrial leptin and leptin receptor expression, and both IL-1 and leptin increase endometrial epithelial β3-integrin expression (Gonzalez et al., 2000). β3-integrin expression is thought to be an important indicator of endometrial receptivity. However, leptin exerts a significantly greater effect on β3 integrin up-regulation than IL-1β at similar concentrations (Gonzalez and Leavis, 2001). Thus, leptin may be involved in preparing the endometrium for embryo implantation. The IL-1β receptor is mainly expressed by endometrial epithelial cells during the luteal phase while IL-1β is expressed by endothelial
and stromal cells throughout the menstrual cycle (Simon et al., 1993). In addition, immunoreactive IL-1β is present in the human maternotrophoblast unit and maternal stromal decidual cells after implantation occurs (Simon et al., 1994). Taken together, these studies suggest that both leptin and IL-1 may play a role in the control of the early stages of human embryo implantation, and therefore dysregulation of either leptin or its receptor or in components of the IL-1 system may cause abnormal embryo implantation and subsequent pregnancy loss.

7.5 IL-11, IL-11Rα mRNA and protein analysis in the endometrium of normal fertile women

The results from these studies indicated that IL-11 mRNA and protein was expressed by both stromal and epithelial cells of the human endometrium throughout the menstrual cycle, agreeing with previous reports (Dimitriadis et al., 2000; Cork et al., 2001; Chen et al., 2002; Karpovich et al., 2003). IL-11 protein expression was greater in epithelial cells than stromal cells throughout the menstrual cycle, agreeing with the results from Cork et al. (2001) and Dimitriadis et al. (2000). IL-11 intensity staining by epithelial cells was maximum in the secretory phase of the cycle, which corresponds to the time of implantation and is when there is maximum expression of other members of this family of cytokines, such as IL-6 and LIF, in the endometrial epithelium (Laird et al., 1997; Von Wolff et al., 2002).

These results also showed that IL-11 mRNA was significantly higher in the late secretory phase compared to the proliferative phase of the menstrual cycle. This increase in IL-11 at this late stage in the cycle is probably due to increased expression in stromal cells, as the immunocytochemistry showed increased protein expression in this compartment at this time, but not in the epithelial compartment. The increased in IL-11 expression by stromal cells during the late secretory phase of the cycle would also suggest a possible function for stromal cell IL-11 in human decidualization process.
IL-11Rα mRNA and protein was also expressed in the human endometrium throughout the menstrual cycle by both stromal and epithelial cells, agreeing with previous reports (Dimitriadis et al., 2000; Cork et al., 2001, 2002; Chen et al., 2002; Karpovich et al., 2003). Similarly to results seen with IL-11, IL-11Rα protein expression was greater in epithelial cells than stromal cells, agreeing with Cork et al. (2001, 2002) and Dimitriadis et al. (2000). IL-11Rα protein expression by both epithelial and stromal cells was slightly increased in the late secretory phase than proliferative phase of the menstrual cycle. This is in contrast to previous studies which were shown no differences in IL-11Rα expression throughout the cycle (Dimitriadis et al., 2000; Chen et al., 2002; Karpovich et al., 2003). However, the menstrual cycle differences seen in our study were not as greater as those seen for IL-11. IL-11Rα mRNA expression by whole tissue was also greater in the secretory phase compared to the proliferative phase of the menstrual cycle. The high levels of IL-11Rα protein in the late secretory phase shown in this study and previous studies, would also support the idea that IL-11 is involved in the functional differentiation that occurs during decidualization of human endometrial stromal cells.

7.6 IL-11, IL-11Rα mRNA and protein analysis in the endometrium of recurrent miscarriage women

We have investigated, for the first time, the expression of IL-11 and IL-11Rα mRNA and protein in the endometrium of women with recurrent miscarriage. Expression was compared between control women and recurrent miscarriage women during the peri-implantation period, as previous studies have shown greatest differences in expression of other endometrial proteins in recurrent miscarriage women and controls at this time (Dalton et al., 1995; Hey et al., 1995). We attempted to compare both mRNA and protein expression for both IL-11 and IL-11Rα. However, RT-PCR products could not be obtained for IL-11 from mRNA extracted from recurrent miscarriage biopsies even using a nested PCR reaction and, therefore, only protein levels could be compared. The lack of success may be due to the smaller size of the biopsies from recurrent miscarriage women and that the quality of the mRNA extracted from them was lower than that obtained from control women.
These results revealed no significant difference in the expression of IL-11Rα mRNA and protein in the endometrium of normal fertile women and recurrent miscarriage women. In contrast to IL-11Rα expression, IL-11 protein expression was decreased in endometrial epithelial cells in the recurrent miscarriage women compared to that seen in normal fertile women. However, there was no significant difference in stromal cell IL-11 expression in the recurrent miscarriage women and normal fertile women. This reduction of IL-11 expression in the endometrium of recurrent miscarriage women is similar to that seen for LIF, IL-6 and IL-1 (Von Wolff et al., 2000; Cork et al., 1999).

7.7 Overall conclusion

In summary this study has shown the presence of IL-11 and IL-11Rα in the endometrium of normal fertile women and recurrent miscarriage women. Epithelial expression of IL-11 is greatest during the mid-secretory phase of the cycle and is decrease in women with recurrent miscarriage, suggesting that it may play a role in implantation. Stromal cells expression of IL-11 and IL-11Rα is greatest in the late secretory phase, suggesting that IL-11 may also play a role in stromal cell decidualisation.

The IL-1RN tandem repeat polymorphism and IL1B-511 polymorphism appear not to be associated with recurrent miscarriage. This does not necessary mean that the IL-1 system is not involved in causing recurrent miscarriage, as the presence of different alleles may not be associated with different levels of IL-1 at the feto-placental unit. Increased 2, 2 genotype frequency for the IL1RN gene polymorphism was seen in recurrent miscarriage women with PCOS. However, the number of women in this group was very low and a larger study on women with PCO disease is required before definitive conclusion can be drawn.

The results from the GLN223ARG leptin receptor polymorphism suggest that leptin may be important in preventing miscarriage in some groups of recurrent miscarriage women (secondary miscarriage & unknown cause). However, it is difficult to see why leptin receptor polymorphisms should be associated with these groups of recurrent miscarriage women. Further studies on large groups of recurrent miscarriage women are required to define the physiological significance of this polymorphism.


Hibi, M., Nakajima, K., Hirano, T. (1996). IL-6 cytokine family and signal transduction: a model of the cytokine system. *Journal of Molecular Medicine.* **74:** 1-12


Karagouni, E.E., Chryssikopoulos, A., Mantzavinos, T., Kanakas, N., Dotsika, E.N. (1998). Interleukin-1β and interleukin-1α may affect the implantation rate of patients undergoing *in vitro* fertilization-embryo transfer. *Fertility & Sterility*. **70**: 553-559


during the menstrual cycle. *Journal of Clinical Endocrinology & Metabolism.* 85: 1946-1950


Korbonits, M., Trainer, P.J., Lit, J.A. (1997). Leptin levels do not change acutely with food administration in normal or obese subjects, but are negatively correlated with pituitary-adrenal activity. *Clinical Endocrinology.* 46: 751-757


Ogasawara, M.I., Aoki, K., Okada, S., Suzumori, K., (2000). Embryonic karyotype of abortuses in relation to the number of previous miscarriages. *Fertility & Sterility.* **73:** 300-304


Raghupathy, R. (1997). Th1-type immunity is incompatible with successful pregnancy. Immunology Today. 18: 478


Read, R. C., Camp, N.J., di Giovine, F. S., Borrow, R., Kaczmarski, E. B.,
associated with fatal outcome of Meningococcal disease. The Journal of
Infections Diseases. 182: 1557-60

Reid, J.G., Simpson, N.A.B., Walker, R.G., Economidou, O., Shillito, J., Good,
cytokine gene polymorphisms in recurrent pregnancy loss. American Journal
of Reproduction Immunology. 45: 35-40

pregnancy wastage. Fertility & Sterility. 39: 123-140

Gynecology. 29: 886-911

Inc., Edinburgh

the gene encoding the murine interleukin-11 receptor α-chain and a related
locus. Journal of Biology & Chemistry. 271: 13754-13761

Robb, L., Li, R., Hartley, L., Nandurkar, H.H., Koentgen, F., Begley, C.G.
(1998). Infertility in female mice lacking the receptor for interleukin-11 is due to a

factor and interleukin-11: cytokines with key roles in implantation. Journal of
Reproductive Immunology. 57: 129-141

Croom Helm Ltd., London.


Wesche, H., Korherr, C., Kracht, M., Falk, W., Resch, K., Martin, M. (1997). The interleukin-1 receptor assessor protein (IL-1RacP) is essential for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP Kinases). *The American Society for Biochemistry and Molecular Biology*. **272**: 7727-7731


Yin, T., Yang, Y.-C. (1994). Mitogen-activated protein kinases and ribosomal S6 protein kinases are involved in signalling pathways shared by interleukin-11, interleukin-6, leukemia inhibitory factor and oncostatin M in mouse 3T3-L1 cells. *Journal of Biology & Chemistry*. **269**: 3731-3738

- 258 -


